frontiers | Research Topics

Burden of diarrhoeal diseases

Edited by
Amir Abdoli, Ali Taghipour, D. Md Robiul Karim
and Milad Badri

Published in
Frontiers in Medicine
Frontiers in Public Health

ISSN 1664-8714
ISBN 978-2-8325-2897-6
DOI 10.3389/978-2-8325-2897-6

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Burden of diarrhoeal diseases

Topic editors

Amir Abdoli — Jahrom University of Medical Sciences, Iran

Ali Taghipour — Jahrom University of Medical Sciences, Iran

D. Md Robiul Karim — Bangabandhu Sheikh Mujibur Rahman Agricultural University, Bangladesh

Milad Badri — Qazvin University of Medical Sciences, Iran

Citation

Abdoli, A., Taghipour, A., Karim, D. M. R., Badri, M., eds. (2023). *Burden of diarrhoeal diseases*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-2897-6

Table of contents

frontiers in Medicine

ORIGINAL RESEARCH
published: 18 November 2021
doi: 10.3389/fmed.2021.776955

Saccharomyces boulardii Combined With Quadruple Therapy for *Helicobacter pylori* Eradication Decreased the Duration and Severity of Diarrhea: A Multi-Center Prospective Randomized Controlled Trial

Yuchong Zhao[1†], Yilei Yang[1†], Aruna[1], Jun Xiao[2], Jun Song[3], Tizheng Huang[4], Shuyu Li[5], Jiguang Kou[6], Lu Huang[7], Dexiong Ji[8], Si Xiong[1], Wang Peng[1], Sanping Xu[9] and Bin Cheng[1*]*

[1] Department of Gastroenterology and Hepatology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [2] Department of Gastroenterology and Hepatology, Zhongnan Hospital of Wuhan University, Wuhan, China, [3] Department of Gastroenterology and Hepatology, Union Hospital (West District), Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [4] Department of Gastroenterology and Hepatology, Hubei Aerospace Hospital, Xiaogan, China, [5] Department of Gastroenterology and Hepatology, The Third People's Hospital of Hubei Province, Wuhan, China, [6] Department of Gastroenterology and Hepatology, The Central Hospital of Xiaogan, Xiaogan, China, [7] Department of Gastroenterology and Hepatology, The First People's Hospital of Xiaogan, Xiaogan, China, [8] Department of Gastroenterology and Hepatology, Anlu People's Hospital, Anlu, China, [9] Department of Gastroenterology and Hepatology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

Background: Whether probiotics helped the *Helicobacter pylori* (*H. pylori*) eradication was still highly controversial. The non-bacterial *Saccharomyces boulardii* (*S. boulardii*) has demonstrated its efficacy in the treatment of antibiotic-associated and infectious diarrhea. We aimed to evaluate the effects of *S. boulardii* combined with quadruple therapy for *H. pylori* eradication and associated side effects.

Methods: Three hundred and sixty *H. pylori*-infected patients were recruited in this multicenter, randomized controlled trial. The patients who underwent *H. pylori* eradication treatment were randomized in a ratio of 1:1 into two separate groups that received standard quadruple therapy (Group A) and quadruple therapy plus *S. boulardii* sachets (Group B) for 14 days. The everyday medication and side-effect records were collected for compliance and adverse effect analysis. All patients accepted $^{13}C/^{14}C$-urea breath tests 4 weeks after the therapy completion.

Results: *Saccharomyces boulardii* and quadruple therapy-combined intervention significantly reduced the incidences of overall side effects (27.8 vs. 38.5%, $p = 0.034$) and diarrhea (11.2 vs. 21.2%, $p = 0.012$) in Group B compared with quadruple therapy alone in Group A, especially reduced the diarrhea duration (5.0 days vs. 7.7 days, $p = 0.032$) and incidence of severe diarrhea (4.7 vs. 10.1%, $p = 0.040$). Intention-to-treat (ITT) analysis and per-protocol (PP) analysis both indicated no statistical differences of

OPEN ACCESS

Edited by:
Marcello Dallio, University of Campania Luigi Vanvitelli, Italy

Reviewed by:
Rinaldo Pellicano, Molinette Hospital, Italy
Yao-Jong Yang, National Cheng Kung University Hospital, Taiwan
Antonietta G. Gravina, University of Campania Luigi Vanvitelli, Italy

***Correspondence:**
Bin Cheng
b.cheng@tjh.tjmu.edu.cn
Sanping Xu
xusanpinghao@aliyun.com

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Gastroenterology, a section of the journal Frontiers in Medicine

Received: *14 September 2021*
Accepted: *04 October 2021*
Published: *18 November 2021*

Citation:
Zhao Y, Yang Y, Aruna, Xiao J, Song J, Huang T, Li S, Kou J, Huang L, Ji D, Xiong S, Peng W, Xu S and Cheng B (2021) Saccharomyces boulardii Combined With Quadruple Therapy for Helicobacter pylori Eradication Decreased the Duration and Severity of Diarrhea: A Multi-Center Prospective Randomized Controlled Trial. Front. Med. 8:776955. doi: 10.3389/fmed.2021.776955

eradication rate between Groups A and B (ITT: 82.7 vs. 85.8%, $p = 0.426$; PP: 89.7 vs. 94.2%, $p = 0.146$). The joint use of *S. boulardii* and quadruple therapy markedly improved the overall pre-eradication alimentary symptoms (hazard ratio (HR): 2.507, 95% CI: 1.449–4.338) recovery.

Conclusion: *Saccharomyces boulardii* ameliorated *H. pylori* eradication-induced antibiotic-associated side effects especially reduced the incidence of severe diarrhea and the duration of diarrhea. However, there was no significant effect of *S. boulardii* on the rate of *H. pylori* eradication.

Trial Registration: The protocol had retrospectively registered at ClinicalTrails.gov, Unique identifier: NCT03688828, date of registration: September 27, 2018; https://clinicaltrials.gov/show/NCT03688828

Keywords: *Helicobacter pylori*, *Saccharomyces boulardii*, eradication, diarrhea, quadruple therapy

INTRODUCTION

The prevalence of *Helicobacter pylori* (*H. pylori*) infection in the general population in China was approximately 60% (1, 2). The eradication of *H. pylori* has been demonstrated effective for alleviating various gastrointestinal (GI) diseases and reducing the risk of gastric cancer (3–6). Current Chinese guidelines recommended 14-day bismuth-containing quadruple therapy as a first-line regimen for *H. pylori* eradication (7). However, due to the increasing resistance to antibiotics and relatively high incidence of side effects, quadruple therapy was not as satisfying as before (8–10). Several previous studies indicated that the use of proton pump inhibitors (PPIs) and antibiotics led to dysbiosis and abundance changes of the gut microbiota (11–13). Though with a relatively low rate of severe side effects, the sporadic reports of *H. pylori* treatment-induced *Clostridium difficile* (*C. difficile*) infection and pseudomembranous colitis have been incessant over the past decades (14–18). Several novel regimens (e.g., high-dose PPI + amoxicillin dual therapy, and vonoprazan-containing therapy) were emerging to conquer the current difficulties, and the preliminary clinical data showed the effect of dual therapy was not inferior to quadruple therapy for *H. pylori* eradication whereas the efficacies of these regimens were restricted by poor compliance and availability (19–21). And the incidence of adverse events (AEs) was nearly equal between the novel treatment and quadruple therapy.

To prevent and treat the underlying AEs brought by *H. pylori* eradication, standard quadruple therapy with probiotic supplements, in particular, *lactobacilli*, *bifidobacterial*, and *Saccharomyces boulardii* (*S. boulardii*) were administrated (22). Non-pathogenic yeast *S. boulardii* was initially used to prevent the *C. difficile* infection and relapse, now it has demonstrated the efficacy of preventing and treating antibiotic-associated, infectious, and functional diarrhea (23, 24). The yeast nature of *S. boulardii* other than bacterial suggested the implications of joint use with antibiotics. Several reports claimed that *S. boulardii* protected intestinal epithelium against pathogen colonization and invasion through upregulating the secretion of sIgA into the luminal mucous, thus exerted the anti-*H. pylori* effect (25–27). However, current clinical studies to investigate the synergistic effect of *S. boulardii* on *H. pylori* infection were highly controversial, even some meta-analyses had contradictory results (28–33). And most of the current trials were about *S. boulardii* and triple therapy combination. Due to the high increasing resistance to antibiotics, triple therapy was no longer effective as used to be. Whether *S. boulardii* could improve the eradication rate of the highly effective 14-day quadruple therapy is still largely unknown.

Most clinical trials about *S. boulardii* focused on the synergetic effect on eradication, the evaluation of diarrhea prevention was usually the secondary aim. Antibiotic-associated diarrhea was the most common AE, ranged from 7.0 to 41.2% (33–38). Though most associated adverse effects were mild and tolerable, diarrhea was the main reason leading to eradication treatment discontinuation. To further investigate the diarrhea prevention and treatment effect of *S. boulardii* in *H. pylori* eradication therapy, we conducted this prospective multicenter randomized controlled trial. Meanwhile, we evaluated the potential synergistic effect of *S. boulardii* on *H. pylori* eradication and alimentary symptoms.

MATERIALS AND METHODS

Patient Enrollment

This study was approved by the Ethical Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (TJ-IRB20180904) and registered on ClinicalTrials.gov (NCT03688828). Between October 2018 and September 2019 from 9 medical centers in China, 360 patients between the ages of 22 and 65 years were enrolled in this study after receiving endoscopic evaluations for various GI symptoms. Each of these patients had $^{13}C/^{14}C$-urea breath test proof of *H. pylori* infection. The exclusion criteria included (1) previous attempts to eradicate *H. pylori*; (2) pregnant or lactation; (3) hypersensitivity to the drugs being used in the study; (4)

Abbreviations: PPI, proton pump inhibitors; GI, gastrointestinal; AEs, adverse events; PP, Per-protocol; ITT, Intention-to-treat; *H. pylori*, *Helicobacter pylori; S. boulardii, Saccharomyces boulardii; C. difficile*, *Clostridium difficile*.

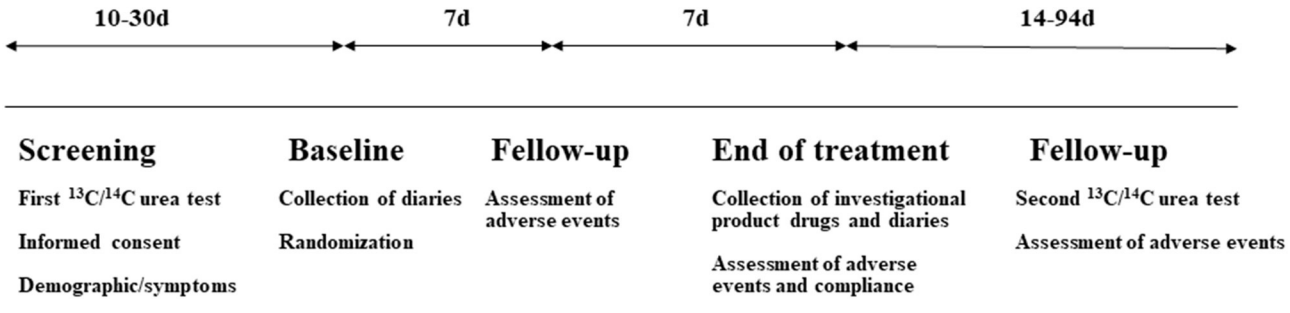

FIGURE 1 | Flowchart of the study progress.

previous treatment with PPIs, bismuth, H2 receptor antagonist, or antibiotics within 4 weeks of the study; and (5) treatment with non-steroidal anti-inflammatory drugs (NSAIDs) or alcohol abuse during the study.

Study Design

This was a randomized, parallel-group study. Three hundred and sixty *H. pylori*-infected patients were recruited in the study and randomly assigned by a computer program into two groups: standard quadruple therapy group (Group A) and quadruple therapy plus *S. bouladii* (Group B). Computer-generated randomization assignments were centralized using the block randomization method (block size of 8) by a data manager who was not involved in the data analysis or patient enrollment. Patients assigned to Group A received esomeprazole (AstraZeneca Pharmaceutical, Co. Ltd., Cambridge, United Kingdom) 20 mg two times a day (*bid*); amoxicillin (Baker Norton Pharmaceutical, Co., Ltd., Kunming, China) 1.0 g *bid*; clarithromycin (Abbott Laboratories Ltd., Shanghai, China) 500 mg bid; and Bismuth Potassium Citrate (Livzon Pharmaceutical Group, Inc., Zhuhai, China) 600 mg *bid* for 14 days. Patients assigned to Group B received the same quadruple therapy for 14 days, Additionally, *S. boulardii* sachets (Laboratories Biocodex, Inc., France) of 500 mg was given a bid to Group B for 14 days. Serious diarrhea patients (mushy stools or watery stools > two times a day) were additionally given montmorillonite powder 3 g *tid*. Considering the high resistance rate of metronidazole in the Center China population, we took amoxicillin and clarithromycin as our antibiotic choice (9, 39).

Study Evaluations and Outcomes

Patients were evaluated at five visits: screening (10–30 days before the baseline visit), baseline, 7 days after the treatment initiation, end of treatment/efficacy (14 days after the treatment initiation), and the second $^{13}C/^{14}C$-urea breath test 4 weeks after the therapy completion and follow-up (44–94 days after treatment completion; **Figure 1**). $^{13}C/^{14}C$-urea breath tests were applied to detect the *H. pylori* infection for the high sensitivity and specificity. Previous studies demonstrated that there were no statistical differences between the ^{13}C and ^{14}C-urea breath tests (40, 41). In this trial, 276 patients accepted the ^{13}C-urea breath test and 84 patients received the ^{14}C-urea breath test. The urea breath test technician was blinded to patient groups.

The primary outcome measure was the incidence of AEs. The investigator will record all AEs related to anti-*H. pylori* therapy, such as nausea, vomiting, taste abnormalities, abdominal pain, abdominal distension, diarrhea, and increased symptoms on the case report form. The incidence of adverse reactions will be assessed at three points: before treatment, during treatment (2 weeks), and after treatment (4–12 weeks). Patients returned their medication and side effects record form after the urea-test re-examination.

The secondary outcome measure was to investigate whether there was a statistical difference in the eradication rate between the two groups. Eradication rate = number of *H. pylori* eradicated cases after treatment/total cases × 100%. Non-ulcer patients will be tested 4 weeks after the end of the eradication treatment, and ulcer patients will be tested 2 weeks after the end of the total course of treatment. Eradication rates were determined by both ITT- and PP-based analyses. All enrolled patients were included in the ITT analysis, but the PP analysis excluded those patients who dropped out due to side effects, loss to follow-up, or poor compliance.

The effect of *S. boulardii* on *H. pylori* eradication rate and the incidence of AEs was also studied using binary logistic regression models, which included the following parameters: eradication rate, the overall incidence of AEs, and the incidence of antibiotic-induced diarrhea.

Sample Size and Statistical Analysis

Based on a literature review of *H. pylori* eradication-induced antibiotic diarrhea (29, 35, 42), we expected a difference between quadruple therapy combined with *S. boulardii* and quadruple therapy alone on the incidence of diarrhea of 13.5 vs. 19.5%. The calculation yielded 179 for combined therapy and 179 for quadruple therapy, with a power of 80% and a two-sided significance level of 0.05 with an assumed 20% dropout rate. Each group should have 184 patients following the randomized block design of eight patients in a group. We calculated a final sample size of 368 patients (184 per group). The full analysis set should be as close as possible to the ITT set. The standards and population

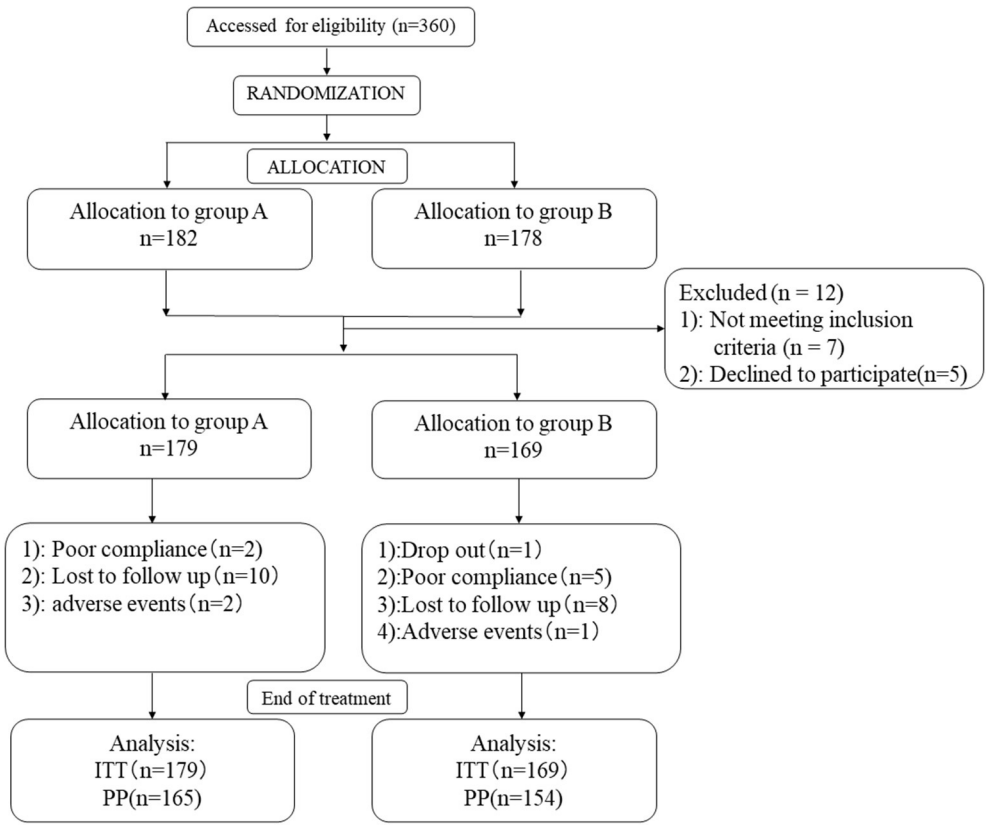

FIGURE 2 | Profile of the study protocol.

of the PP set will be finalized after data-blinding verification. The direct deletion method will be used to treat missing data.

In this study, the demographic and clinical characteristics of the patients will be summarized with mean and SD. The results of eradication and incidence of AEs are expressed in terms of the number of cases and percentage.

Qualitative variables were compared using the chi-squared test and Fisher's exact test, while Student's *t*-test and the Mann-Whitney *U* test were used for quantitative variables. The effect of *S. boulardii* plus sequential therapy on the eradication rate and the incidence of antibiotic-induced AEs were determined using binary logistic regression, and p-value < 0.05 with two-tail will be considered significant. All statistical analyses will be performed by blinded professional statisticians using SPSS V.26.0.

RESULTS

Patient Disposition and Characteristics

A total of 348 patients fulfilling the inclusion criteria were enrolled in this trial, with 179 patients in Group A and 169 patients in Group B for ITT analysis. Twenty-nine patients (8.33%) were excluded from PP analysis. Follow-up was incomplete in 10 patients (5.6%) and 8 patients (4.7%) in Groups A and B, respectively. Two patients in Group A discontinued treatment because of severe diarrhea while one patient in Group B discontinued for skin rash. Poor treatment compliance was reported in two (1.1%) patients and five (3.0%) patients in Groups A and B. Apart from this, there was one patient who dropped out from treatment in group B because of pregnancy (**Figure 2**). At baseline, there were no statistically significant differences in the baseline characteristics of patients included in the two study groups (**Table 1**).

Efficacy Analysis

Intention-to-treat analysis demonstrated that the eradication rates were 85.8% for Group B and 82.7% for Group A (hazard ratio, HR, = 1.038, $p = 0.426$, 95% CI = 0.948–1.136). PP analysis indicated that the eradication results were 89.7% for Group B and 94.2% for Group A (HR = 1.851, $p = 0.146$, 95% CI = 0.799–4.286; **Table 2**). Both ITT and PP analyses showed no statistical differences in the eradication rate between Groups A and B.

The follow-up analysis of the alimentary symptoms, from pre-treatment of 3 months after the eradication, indicated that the overall symptoms improvement rate of Group B (78.6 vs. 58.3%, $p < 0.001$) was significantly higher than that of Group A (**Table 2**). Further analysis showed that the abdominal distension recovery

TABLE 1 | Baseline characteristics of all patients.

Characteristics	Group A ($n = 179$)	Group B ($n = 169$)	*P*-value
Age, mean ± SD, years	46.68±12.85	45.31±11.46	0.308
Sex, % of female	45.7%	49.1%	0.536
Endoscopic findings, *n* (%)			
Non-atrophic gastritis	47 (26.3%)	40 (23.7%)	0.577
Atrophic gastritis	21 (11.7%)	27 (16.0%)	0.251
Ulcer	32 (17.9%)	33 (19.5%)	0.693
Rural/urban, % of rural	70.4%	68.0%	0.636
Pre-treatment symptoms, *n* (%)	127 (75.1%)	126 (70.4%)	0.321
Epigastric pain	63 (37.3%)	54 (30.2%)	0.161
Epigastric distending	66 (39.1%)	66 (36.9%)	0.676
Regurgitation/heartburn	34 (20.1%)	43 (25.4%)	0.382
Frequent belching	29 (17.2%)	20 (11.2%)	0.109
Nausea/vomiting	19 (11.2%)	15 (8.4%)	0.370

TABLE 2 | Comparison of clinical therapeutic effect analysis between two groups.

Therapeutic effect	Group A	Group B	*P*-value
Eradication rate			
ITT	148/179 (82.7%)	145/169 (85.8%)	0.426
PP	148/165 (89.7%)	145/154 (94.2%)	0.146
3-month symptom complete relief rate	74/127 (58.3%)	98/126 (78.6%)	<0.001***
Epigastric pain	53/63 (81.5%)	50/54 (75.8%)	0.162
Abdominal distension	35/66 (53.0%)	49/66 (89.4%)	0.011*
REGURGITATION/heartburn	26/34 (76.5%)	35/43 (81.4%)	0.603
Frequent belching	21/29 (72.4%)	16/20 (80.0%)	0.122
Nausea/vomiting	14/19 (74.7%)	13/15 (86.7%)	0.368

*ITT, intention-to-treat analysis; PP, per-protocol analysis. *$p < 0.05$, significant difference, ***$p < 0.001$.*

rate in Group B compared was markedly higher than in Group A (89.4 vs. 53.0%, $p = 0.011$).

Side Effects Analysis

The overall incidences of AEs in the experimental and control groups were 38.5 and 27.8%, respectively, representing a decrease of 10.7% in the experimental group ($p = 0.034$). The diarrhea rate of Group A was significantly higher than that of Group B (21.2 vs. 11.2%, $p = 0.012$). Meanwhile, the combination of *S. boulardii* and quadruple therapy decreased the duration of diarrhea (5.0 days vs. 7.7 days, $p = 0.032$) and incidence of severe diarrhea (10.1 vs. 4.7%, $p = 0.040$) in Group B compared with Group A (**Figure 3**). There were no statistical differences between Groups A and B in terms of vomiting, constipation, or allergy (**Table 3**). Results of the multivariate analysis further verified that the combination of *S. boulardii* and quadruple treatment reduced the overall incidence of AEs (odds ratio, OR: 0.378, 95% CI: 0.117–0.807) and incidence of diarrhea (OR: 0.359, 95% CI: 0.148–0.872) compared with quadruple therapy alone (**Table 4**). Two patients in Group A accepted intravenous (*i.v.*) treatment for severe diarrhea other than montmorillonite powder and discontinued the eradication therapy. However, no *C. difficile* was detected by fecal examination. And no patients in Group B discontinued the therapy due to diarrhea.

DISCUSSION

The synergetic effect of *S. boulardii* on *H. pylori* eradication and associated side effects were analyzed in this study through the first multicenter randomized controlled trials of *S. boulardii* and quadruple therapy combination in China. We demonstrated that the administration of *S. boulardii* significantly decreased the incidence of eradication-associated AEs (OR: 0.378, 95% CI: 0.117–0.807), especially reduced the duration of diarrhea (5.0 days vs. 7.7 days, $p = 0.032$) and incidence of severe diarrhea (4.7 vs. 10.1%, $p = 0.040$). However, *S. boulardii* did not improve the eradication rate for bismuth quadruple therapy. However, the joint use of *S. boulardii* significantly improved gastritis/ulcer-associated symptoms (HR: 2.507, 95% CI: 1.449–4.338).

Unlike synergetic effects of *H. pylori* eradication, the effect of probiotics on AEs prevention and treatment was definite. Distinct from other bacterial probiotics, *S. boulardii* is a non-pathogenic fungus resistant to gastric acid and antibiotics, thus it could be used with eradication therapy simultaneously (24, 25). *S. boulardii* was used for the prevention of *C. difficile* infection originally (43). With sporadic reports of pseudomembranous colitis during *H. pylori* eradication, *S. boulardii* was gradually used as a supplement for the prevention and treatment of AEs. Acute severe diarrhea was the main reason for eradication therapy discontinuation thus leading to treatment failure. And some reports claimed that the diarrhea prevention effect of *S. boulardii* was only notable in children but not in adults (35, 44). Although various trials have already demonstrated the efficacy of *S. boulardii* to prevent and treat antibiotic-associated diarrhea (33, 35, 45, 46). Our study refined the diarrhea-associated data and verified that *S. boulardii* reduced the diarrhea duration and incidence of severe diarrhea correlated with eradication therapy. None of the diarrhea patients in the quadruple therapy plus *S. boulardii* group required further diarrhea treatment, whereas two patients in the quadruple therapy alone group accepted intravenous fluid infusion.

Probiotics might be effective for improving *H. pylori* eradication rates due to the decrease in the AEs and potential mucosal barrier protective effect (29, 36, 37, 47). Previous studies demonstrated the probiotics exerted the synergetic eradication effect through a similar mechanism including competitively inhibiting the *H. pylori* adhesion to gastric mucosa (48, 49) or producing antimicrobial molecules (29, 50). Recently, Yang et al. reported that the administration of *S. boulardii* could inhibit the *H. pylori* infection-induced gastric lymphoid follicle formation (26). Previous experiments mainly adopted the combination of *S. boulardii* and triple therapy. However, due to high resistance and decreasing efficacy, triple therapy was no

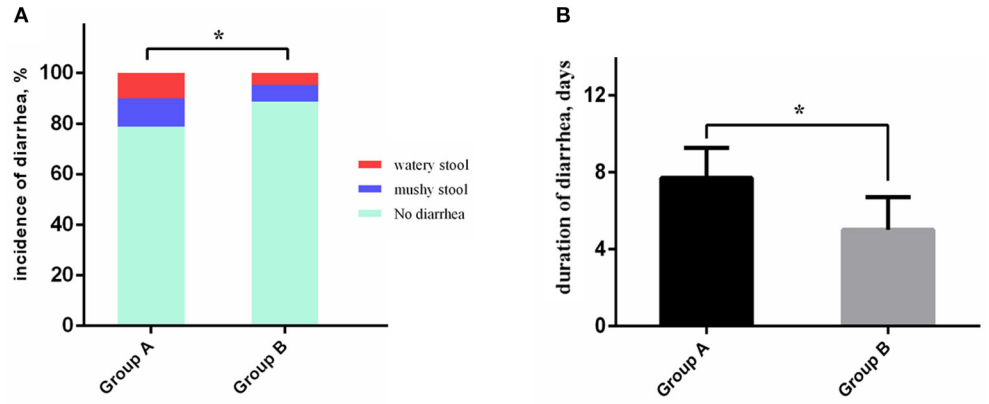

FIGURE 3 | Antibiotic-associated diarrhea characteristics comparison between the two groups. **(A)** The severe diarrhea patients and overall diarrhea patients occupy a bigger proportion in Group A than in Group B. **(B)** The duration of diarrhea of patients in Group B was significantly shorter than in Group A. *$P < 0.05$.

TABLE 3 | Comparison of incidence of AEs between two groups.

Adverse events	Group A	Group B	*P*-value
Overall adverse events, n (%)	69 (38.5%)	47 (27.8%)	0.034*
Vomiting	20 (11.2%)	27 (15.9%)	0.190
Diarrhea	38 (21.2%)	19 (11.2%)	0.012*
Duration of diarrhea ± SEM, days	7.7 (0.7)	5.0 (0.8)	0.032*
Watery stools	18 (10.1%)	8 (4.7%)	0.040*
Mushy stools	20 (11.2%)	11 (6.5%)	
Allergy	3 (1.7%)	1 (0.6%)	0.623
Constipation	6 (3.4%)	5 (3.0%)	0.834

*SEM, standard error of mean; AEs, adverse events. *p < 0.05, significant difference.*

TABLE 4 | Comparison of incidence of AEs among two groups.

Model	Group A		Group B	
	OR (95% CI)	*P* -value	OR (95% CI)	*P*-value
Incidence of overall AE				
Crude model	1.000 (*ref.*)	-	0.614 (0.391–0.965)	0.034*
Model 1	1.000 (*ref.*)	-	0.643 (0.399–1.006)	0.053
Model 2	1.000 (*ref.*)	-	0.378 (0.117–0.807)	0.012*
Incidence of diarrhea				
Crude model	1.000 (*ref.*)	-	0.470 (0.259–0.854)	0.013*
Model 1	1.000 (*ref.*)	-	0.486 (0.265–0.893)	0.020*
Model 2	1.000 (*ref.*)	-	0.359 (0.148–0.872)	0.024*

*Statisitical analyses are based on a multivariable logistic regression model. AEs, adverse events. Model 1 was adjusted for age and sex. Model 2 was additionally adjusted for rural/urban, endoscopic features, and different medical centers. *p < 0.05, significant difference.*

longer the first-line recommendation for *H. pylori* eradication in China (7). The eradication rate for bismuth quadruple therapy has already reached 87.6–92.6% (51). In addition, the synergetic effect of *S. boulardii* on eradication was highly controversial for many years even with low-efficacy triple therapy. Some meta-analyses gave contradictory results, let alone some prospective and respective trials. Szayewska et al. reported in a meta-analysis that *S. boulardii* improved the eradication rate [Risk Ratio (RR): 1.11, 95% CI: 1.06–1.17; moderate-quality evidence] whereas Wang et al. found no better efficacy of any probiotic supplement for *H. pylori* eradication (24, 29). This is the first multicenter randomized controlled trial for *S. boulardii* and bismuth quadruple therapy in China. And our study showed that this probiotic supplement barely improved the eradication rate of quadruple therapy. Considering the eradication rate was still at a satisfying level compared with triple therapy, no improvement after the *S. boulardii* supplement became reasonable. It was a novel finding that joint administration of *S. boulardii* significantly improved the original dyspepsia symptoms.

Collectively, our study demonstrated that *S. boulardii*-ameliorated *H. pylori* eradication-induced antibiotic-associated diarrhea especially decreased the diarrhea duration and the incidence of severe diarrhea. Different from the combination with triple therapy, *S. boulardii* did not affect the quadruple therapy eradication rate of *H. pylori*, whereas improved the pre-treatment dyspepsia symptoms.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. The patients/participants

provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BC and SXu designed the study. YZ and YY performed data analysis. SXi, YZ, A, JX, SL, JK, TH, LH, and DJ performed the acquisition of data. YZ and YY drafted the manuscript. SXi provided critical revision of the manuscript for important intellectual content. YZ, SXi, and YY performed technical support. BC and SXu performed study supervision. All authors have read and approved the manuscript.

FUNDING

This work was supported by the National Natural Science Foundation of China Grant Nos. 81802427 (to ZL), 81372352 (to BC).

ACKNOWLEDGMENTS

We sincerely thank Dr. Ping Han and Dr. Zhou Luan from the Tongji Hospital, Tongji Medical College, HUST, and Dr. Liangkai Chen and Prof. Ping Yin from the Department of Biostatistics, School of Public Health, Tongji Medical College for their efforts.

REFERENCES

1. Hooi JKY, Lai WY, Ng WK, Suen MMY, Underwood FE, Tanyingoh D, et al. Global prevalence of helicobacter pylori infection: systematic review and meta-analysis. *Gastroenterology.* (2017) 153:420–9. doi: 10.1053/j.gastro.2017.04.022
2. Nagy P, Johansson S, Molloy-Bland M. Systematic review of time trends in the prevalence of Helicobacter pylori infection in China and the USA. *Gut Pathog.* (2016) 8:8. doi: 10.1186/s13099-016-0091-7
3. Wong BC, Lam SK, Wong WM, Chen JS, Zheng TT, Feng RE, et al. Helicobacter pylori eradication to prevent gastric cancer in a high-risk region of China: a randomized controlled trial. *JAMA.* (2004) 291:187. doi: 10.1001/jama.291.2.187
4. Sugano K, Tack J, Kuipers EJ, Graham DY, El-Omar EM, Miura S, et al. Kyoto global consensus report on Helicobacter pylori gastritis. *Gut.* (2015) 64:1353–67. doi: 10.1136/gutjnl-2015-309252
5. Fallone CA, Chiba N, Zanten SVV, Fischbach L, Gisbert JP, Hunt RH, et al. The toronto consensus for the treatment of helicobacter pylori infection in adults. *Gastroenterology.* (2016) 151:51–69.e14. doi: 10.1053/j.gastro.2016.04.006
6. Malfertheiner P, Megraud F, O'Morain CA, Gisbert JP, Kuipers EJ, Axon AT, et al. Management of helicobacter pylori infection-the maastricht V/Florence consensus report. *Gut.* (2017) 66:6–30. doi: 10.1136/gutjnl-2016-312288
7. Liu WZ, Xie Y, Lu H, Cheng H, Zeng ZR, Zhou LY, et al. Fifth chinese national consensus report on the management of helicobacter pylori infection. *Helicobacter.* (2018) 23:e12475. doi: 10.1111/hel.12475
8. Kuo YT, Liou JM, El-Omar EM, Wu JY, Leow AHR, Goh KL, et al. Primary antibiotic resistance in Helicobacter pylori in the Asia-Pacific region: a systematic review and meta-analysis. *Lancet Gastroenterol Hepatol.* (2017) 2:707–15. doi: 10.1016/S2468-1253(17)30219-4
9. Hu Y, Zhu Y, Lu NH. Primary Antibiotic Resistance of Helicobacter pylori in China. *Digest Dis Sci.* (2017) 62:1–9. doi: 10.1007/s10620-017-4536-8
10. Fallone CA, Moss SF, Malfertheiner P. Reconciliation of recent helicobacter pylori treatment guidelines in a time of increasing resistance to antibiotics. *Gastroenterology.* (2019) 157:44–53. doi: 10.1053/j.gastro.2019.04.011
11. Hsu PI, Pan CY, Kao JY, Tsay FW, Peng NJ, Kao SS, et al. Helicobacter pylori eradication with bismuth quadruple therapy leads to dysbiosis of gut microbiota with an increased relative abundance of Proteobacteria and decreased relative abundances of Bacteroidetes and Actinobacteria. *Helicobacter.* (2018) 23:e12498. doi: 10.1111/hel.12498
12. Oh B, Kim BS, Kim JW, Kim JS, Koh SJ, Kim BG, et al. The effect of probiotics on gut microbiota during the helicobacter pylori eradication: randomized controlled trial. *Helicobacter.* (2016) 21:165–74. doi: 10.1111/hel.12270
13. Jackson MA, Goodrich JK, Maxan ME, Freedberg DE, Abrams JA, Poole AC, et al. Proton pump inhibitors alter the composition of the gut microbiota. *Gut.* (2016) 65:749–56. doi: 10.1136/gutjnl-2015-310861
14. Nawaz A, Mohammed I, Ahsan K, Karakurum A, Hadjiyane C, Pellecchia C. Clostridium difficile colitis associated with treatment of Helicobacter pylori infection. *Am J Gastroenterol.* (1998) 93:1175–6. doi: 10.1111/j.1572-0241.1998.00358.x
15. Nei T, Hagiwara J, Takiguchi T, Yokobori S, Shiei K, Yokota H, et al. Fatal fulminant Clostridioides difficile colitis caused by Helicobacter pylori eradication therapy a case report. *J Infect Chemother.* (2020) 26:305–8. doi: 10.1016/j.jiac.2019.10.021
16. Trifan A, Girleanu I, Cojocariu C, Sfarti C, Singeap AM, Dorobat C, et al. Pseudomembranous colitis associated with a triple therapy for Helicobacter pylori eradication. *World J Gastroenterol.* (2013) 19:7476–9. doi: 10.3748/wjg.v19.i42.7476
17. Sato S, Chinda D, Yamai K, Satake R, Soma Y, Shimoyama T, et al. A case of severe pseudomembranous colitis diagnosed by colonoscopy after Helicobacter pylori eradication. *Clin J Gastroenterol.* (2014) 7:247–50. doi: 10.1007/s12328-014-0490-6
18. Prieto de. Paula JM, García Colodro J, Prieto Dehesa M, Franco Hidalgo S. Clostridium difficile infection associated with metronidazole-based treatment for Helicobacter pylori eradication. *Gastroenterol Hepatol.* (2019) 42:524. doi: 10.1016/j.gastre.2019.05.005
19. Gao W, Ye H, Deng X, Wang C, Xu Y, Li Y, et al. Rabeprazole-amoxicillin dual therapy as first-line treatment for H pylori eradication in special patients: a retrospective, real-life study. *Helicobacter.* (2020) 25:e12717. doi: 10.1111/hel.12717
20. Yu L, Luo L, Long X, Liang X, Ji Y, Graham DY, et al. High-dose PPI-amoxicillin dual therapy with or without bismuth for first-line Helicobacter pylori therapy: a randomized trial. *Helicobacter.* (2019) 24:e12596. doi: 10.1111/hel.12596
21. Suzuki S, Gotoda T, Kusano C, Ikehara H, Ichijima R, Ohyauchi M, et al. Seven-day vonoprazan and low-dose amoxicillin dual therapy as first-line Helicobacter pylori treatment: a multicentre randomised trial in Japan. *Gut.* (2020) 69:1019–26. doi: 10.1136/gutjnl-2019-319954
22. Currò D, Ianiro G, Pecere S, Bibbò S, Cammarota G. Probiotics, fibre and herbal medicinal products for functional and inflammatory bowel disorders. *Br J Pharmacol.* (2017) 174:1426–49. doi: 10.1111/bph.13632
23. Cárdenas PA, Garcés D, Prado-Vivar B, Flores N, Fornasini M, Cohen H, et al. Effect of Saccharomyces boulardii CNCM I-745 as complementary treatment of Helicobacter pylori infection on gut microbiome. *Eur J Clin Microbiol Infect Dis.* (2020) 39:1365–72. doi: 10.1007/s10096-020-03854-3
24. Szajewska H, Horvath A, Kołodziej M. Systematic review with meta-analysis: Saccharomyces boulardii supplementation and eradication of Helicobacter pylori infection. *Aliment Pharmacol Ther.* (2015) 41:1237–45. doi: 10.1111/apt.13214
25. Sakarya S, Gunay N. Saccharomyces boulardii expresses neuraminidase activity selective for α2,3-linked sialic acid that decreases Helicobacter pylori adhesion to host cells. *APMIS.* (2014) 122:941–50. doi: 10.1111/apm.12237
26. Yang L, Tian ZB, Yu YN, Zhang CP, Li XY, Mao T, et al. Saccharomyces boulardii administration can inhibit the formation of gastric lymphoid follicles induced by Helicobacter suis infection. *Pathogen Dis.* (2017) 75:ftx006. doi: 10.1093/femspd/ftx006
27. Rodrigues AC, Cara DC, Fretez SH, Cunha FQ, Vieira EC, Nicoli JR, et al. Saccharomyces boulardii stimulates sIgA production and the phagocytic system of gnotobiotic mice. *J Appl Microbiol.* (2000) 89:404–14. doi: 10.1046/j.1365-2672.2000.01128.x

28. Chang YW, Park YM, Oh CH, Oh SJ, Cho JH, Kim JW, et al. Effects of probiotics or broccoli supplementation on Helicobacter pylori eradication with standard clarithromycin-based triple therapy. *Korean J Intern Med.* (2020) 35:574–81. doi: 10.3904/kjim.2019.139
29. Wang F, Feng J, Chen P, Liu X, Ma M, Zhou R, et al. Probiotics in Helicobacter pylori eradication therapy: Systematic review and network meta-analysis. *Clin Res Hepatol Gastroenterol.* (2017) 41:466–75. doi: 10.1016/j.clinre.2017.04.004
30. Zojaji H, Ghobakhlou M, Rajabalinia H, Ataei E, Jahani SS, Moghimidehkordi B, et al. The efficacy and safety of adding the probiotic Saccharomyces boulardiito standard triple therapy for eradication of Hpylori: a randomized controlled trial. *Gastroenterol Hepatol Bed Bench.* (2013) 6:S99–S104.
31. Szajewska H, Horvath A, Piwowarczyk A. Meta-analysis: the effects of Saccharomyces boulardii supplementation on Helicobacter pylori eradication rates and side effects during treatment. *Aliment Pharmacol Ther.* (2010) 32:1069–79. doi: 10.1111/j.1365-2036.2010.04457.x
32. Hurduc V, Plesca D, Dragomir D, Sajin M, Vandenplas Y. A randomized, open trial evaluating the effect of Saccharomyces boulardii on the eradication rate of Helicobacter pylori infection in children. *Acta Pădiatrica.* (2009) 98:127–31. doi: 10.1111/j.1651-2227.2008.00977.x
33. Cindoruk M, Erkan G, Karakan T, Dursun A, Unal S. Efficacy and safety of Saccharomyces boulardii in the 14-day triple anti-Helicobacter pylori therapy: a prospective randomized placebo-controlled double-blind study. *Helicobacter.* (2007) 12:309–16. doi: 10.1111/j.1523-5378.2007.00516.x
34. Kwon SB, Lee KL, Kim JS, Lee JK, Kim W, Jung YJ, et al. Antibiotics-associated diarrhea and other gastrointestinal abnormal responses regarding Helicobacter pylori eradication. *Korean J Gastroenterol.* (2010) 56:229–35. doi: 10.4166/kjg.2010.56.4.229
35. Szajewska H, Kołodziej M. Systematic review with meta-analysis: saccharomyces boulardii in the prevention of antibiotic-associated diarrhoea. *Aliment Pharmacol Ther.* (2015) 42:793–801. doi: 10.1111/apt.13344
36. Çekin AH, Sahintürk Y, Akbay Harmandar F, Uyar S, Yolcular BO, Çekin Y. Use of probiotics as an adjuvant to sequential H. pylori eradication therapy: impact on eradication rates, treatment resistance, treatment-related side effects, and patient compliance. *Turkish J Gastroenterol.* (2017) 28:3–11. doi: 10.5152/tjg.2016.0278
37. de Vrese M, Kristen H, Rautenberg P, Laue C, Schrezenmeir J. Probiotic lactobacilli and bifidobacteria in a fermented milk product with added fruit preparation reduce antibiotic associated diarrhea and Helicobacter pylori activity. *J Dairy Res.* (2011) 78:396–403. doi: 10.1017/S002202991100063X
38. Nyssen OP, Perez-Aisa A, Tepes B, Castro-Fernandez M, Kupcinskas J, Jonaitis L, et al. Adverse event profile during the treatment of helicobacter pylori: a real-world experience of 22,000 patients from the European Registry on H. pylori Management (Hp-EuReg). *Am J Gastroenterol.* (2021) 116:1220–9. doi: 10.14309/ajg.0000000000001246
39. Liu DS, Wang YH, Zeng ZR, Zhang ZY, Lu H, Xu JM, et al. Primary antibiotic resistance of Helicobacter pylori in Chinese patients: a multiregion prospective 7-year study. *Clin Microbiol Infect.* (2018) 24:780.e5–e8. doi: 10.1016/j.cmi.2017.11.010
40. Hart GC, Avison MP. 13C-urea versus 14C-urea breath test: is there still a need for 14C-urea? *Nucl Med Commun.* (1999) 20:495–6. doi: 10.1097/00006231-199905000-00141
41. Best LM, Takwoingi Y, Siddique S, Selladurai A, Gandhi A, Low B, et al. Non-invasive diagnostic tests for Helicobacter pylori infection. *Cochrane Datab System Rev.* (2018) 3:Cd012080. doi: 10.1002/14651858.CD012080.pub2
42. McFarland LV, Huang Y, Wang L, Malfertheiner P. Systematic review and meta-analysis: Multi-strain probiotics as adjunct therapy for Helicobacter pylori eradication and prevention of adverse events. *United Eur Gastroenterol J.* (2016) 4:546–61. doi: 10.1177/2050640615617358
43. Mcfarland LV, Surawicz CM, Greenberg RN, Fekety R, Elmer GW, Moyer KA, et al. A randomized placebo-controlled trial of saccharomyces boulardii in combination with standard antibiotics for clostridium difficile disease. *JAMA.* (1994) 271:1913–8. doi: 10.1001/jama.271.24.1913
44. Mcfarland LV. Systematic review and meta-analysis of Saccharomyces boulardii in adult patients. *World J Gastroenterol.* (2010) 16:2202–22. doi: 10.3748/wjg.v16.i18.2202
45. Feizizadeh S, Salehi-Abargouei A, Akbari V. Efficacy and safety of Saccharomyces boulardii for acute diarrhea. *Pediatrics.* (2014) 134:e176–91. doi: 10.1542/peds.2013-3950
46. Surawicz CM, Elmer GW, Speelman P, Mcfarland LV, Chinn J, Van BG. Prevention of antibiotic-associated diarrhea by Saccharomyces boulardii: a prospective study. *Zeitschrift Für Gastroenterol.* (1989) 96:981–8. doi: 10.1016/0016-5085(89)91613-2
47. Song MJ, Dongil P, Jungho P, Hongjoo K, Yongkyun C, Chongil S, et al. The effect of probiotics and mucoprotective agents on PPI-based triple therapy for eradication of Helicobacter pylori. *Gastroenterology.* (2010) 138:S335. doi: 10.1111/j.1523-5378.2010.00751.x
48. Mukai T, Asasaka T, Sato E, Mori K, Matsumoto M, Ohori H. Inhibition of binding of Helicobacter pylori to the glycolipid receptors by probiotic Lactobacillus reuteri. *FEMS Immunol Med Microbiol.* (2002) 32:105–10. doi: 10.1111/j.1574-695X.2002.tb00541.x
49. Homan M, Orel R. Are probiotics useful in Helicobacter pylori eradication? *World J Gastroenterol.* (2015) 21:10644–53. doi: 10.3748/wjg.v21.i37.10644
50. Kim TS, Hur JW Yu MA, Cheigh CI, Kim KN, Hwang JK, et al. Antagonism of Helicobacter pylori by bacteriocins of lactic acid bacteria. *J Food Prot.* (2003) 66:3–12. doi: 10.4315/0362-028X-66.1.3
51. Liou JM, Fang YJ, Chen CC, Bair MJ, Chang CY, Lee YC, et al. Concomitant, bismuth quadruple, and 14-day triple therapy in the first-line treatment of Helicobacter pylori: a multicentre, open-label, randomised trial. *Lancet.* (2016) 388:2355–65. doi: 10.1016/S0140-6736(16)31409-X

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Medicine

ORIGINAL RESEARCH
published: 07 January 2022
doi: 10.3389/fmed.2021.786096

Study of Risk Factors for Total Attack Rate and Transmission Dynamics of Norovirus Outbreaks, Jiangsu Province, China, From 2012 to 2018

Jing Ai[1,2†], Yuanzhao Zhu[3†], Jianguang Fu[1,2†], Xiaoqing Cheng[1,2†], Xuefeng Zhang[1,2], Hong Ji[1,2], Wendong Liu[1,2], Jia Rui[3], Jingwen Xu[3], Tianlong Yang[3], Yao Wang[3], Xingchun Liu[3], Meng Yang[3], Shengnan Lin[3], Xiaohao Guo[3], Changjun Bao[1,2], Qun Li[4*] and Tianmu Chen[3*]*

[1] Jiangsu Provincial Center for Disease Control and Prevention, Nanjing, China, [2] Key Laboratory of Enteric Pathogenic Microbiology, Ministry of Health, Jiangsu Provincial Center for Disease Control and Prevention, Nanjing, China, [3] State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics, School of Public Health, Xiamen University, Xiamen, China, [4] Public Health Emergency Center, Chinese Center for Disease Control and Prevention, Beijing, China

Objective: To describe the epidemiological characteristics of norovirus outbreaks in Jiangsu Province, utilize the total attack rate (*TAR*) and transmissibility (R_{unc}) as the measurement indicators of the outbreak, and a statistical difference in risk factors associated with *TAR* and transmissibility was compared. Ultimately, this study aimed to provide scientific suggestions to develop the most appropriate prevention and control measures.

Method: We collected epidemiological data from investigation reports of all norovirus outbreaks in Jiangsu Province from 2012 to 2018 and performed epidemiological descriptions, sequenced the genes of the positive specimens collected that were eligible for sequencing, created a database and calculated the *TAR*, constructed SEIAR and SEIARW transmission dynamic models to calculate R_{unc}, and performed statistical analyses of risk factors associated with the *TAR* and R_{unc}.

Results: We collected a total of 206 reported outbreaks, of which 145 could be used to calculate transmissibility. The mean *TAR* in was 2.6% and the mean R_{unc} was 12.2. The epidemiological characteristics of norovirus outbreaks showed an overall increasing trend in the number of norovirus outbreaks from 2012 to 2018; more outbreaks in southern Jiangsu than northern Jiangsu; more outbreaks in urban areas than in rural areas; outbreaks occurred mostly in autumn and winter. Most of the sites where outbreaks occurred were schools, especially primary schools. Interpersonal transmission accounted for the majority. Analysis of the genotypes of noroviruses revealed that the major genotypes of the viruses changed every 3 years, with the GII.2 [P16] type of norovirus dominating from 2016 to 2018. Statistical analysis of *TAR* associated with risk factors found statistical differences in all risk factors, including time (year, month, season), location (geographic location, type of settlement, type of premises), population (total number of susceptible people at the outbreak site), transmission route, and genotype

OPEN ACCESS

Edited by:
Amir Abdoli, Jahrom University of Medical Sciences, Iran

Reviewed by:
Leena Maunula, University of Helsinki, Finland
Eric Levenson, United States Food and Drug Administration, United States

***Correspondence:**
Changjun Bao
bao2000_cn@163.com
Qun Li
liqun@chinacdc.cn
Tianmu Chen
13698665@qq.com

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *29 September 2021*
Accepted: *01 December 2021*
Published: *07 January 2022*

Citation:
Ai J, Zhu Y, Fu J, Cheng X, Zhang X, Ji H, Liu W, Rui J, Xu J, Yang T, Wang Y, Liu X, Yang M, Lin S, Guo X, Bao C, Li Q and Chen T (2022) Study of Risk Factors for Total Attack Rate and Transmission Dynamics of Norovirus Outbreaks, Jiangsu Province, China, From 2012 to 2018. Front. Med. 8:786096. doi: 10.3389/fmed.2021.786096

($P < 0.05$). Statistical analysis of transmissibility associated with risk factors revealed that only transmissibility was statistically different between sites.

Conclusions: The number of norovirus outbreaks in Jiangsu Province continues to increase during the follow-up period. Our findings highlight the impact of different factors on norovirus outbreaks and identify the key points of prevention and control in Jiangsu Province.

Keywords: infectious diseases, norovirus, total attack rate, transmission dynamics, risk factors

INTRODUCTION

Norovirus, initially known as winter vomiting disease, was identified as the cause of the outbreak in 1972 when researchers observed 27-nm-virus particles by immune electron microscopy in the infected fecal filtrate from an acute outbreak of gastroenteritis in Norwalk, Ohio, in 1968, and named "Norwalk virus" (1, 2). Norovirus is a single-stranded and unenveloped RNA virus, which can be classified into 10 genogroups (GI-GX) and 49 genotypes with most of the infections in humans caused by GI and GII genogroups, which could be further divided to 9 and 27 genotypes, respectively (3). Currently, no drugs effectively treat norovirus.

The disease burden of norovirus is becoming increasingly serious worldwide and in China, causing ∼684 million cases and 212,000 deaths, annually (4). Compared with other countries, China has a higher prevalence of norovirus (19.8–21.0%) (5). The annual report of the National Statutory Infectious Diseases Reporting System, found a significant decrease in the incidence of infectious diarrhea caused by cholera, dysentery and typhoid fever between 2006 and 2016; on the other hand, the number of cases caused by viral pathogens increased from 741,809 to 1,017,962 (6). Nowadays, norovirus is replacing rotavirus as the main pathogen of viral acute gastroenteritis, which indicates that norovirus outbreaks remain an important public health problem in China. Understanding the characteristics of the disease is key to the effective implementation of disease prevention and control. Existing studies have found significant differences in the epidemiological characteristics of norovirus outbreaks between China and other countries. First, whole populations are susceptible to norovirus infection (7); however, the outbreak sites are different. In developed countries, norovirus outbreaks occur mainly in medical institutions, such as long-term care facilities and hospitals (8, 9), whereas in China, norovirus outbreaks mainly occur in school environments and nurseries (10–13). Second, different age groups face different risks of infection; particularly, one study found that the highest incidence was observed in children aged <5 years (14). Other studies have found that children younger than 12 years are more susceptible and infection is common in children under 2 years old in developing countries (15, 16). Additionally, differences were observed between the genotypes. In the past two decades, the GII.4 virus has caused norovirus outbreaks in most age groups worldwide (17). However, norovirus genotypes are constantly changing in China with the emergence of new genotypes and differences in genotypes observed in different regions.

In recent years, an increase has been observed in the incidence rate of infectious diarrhea in Jiangsu Province. In a study on norovirus and meteorological factors in Jiangsu Province, bacterial culture and viral nucleic acid testing were performed on 6,640 stool specimens collected, and 1,193 positive specimens were obtained, of which the positive rate for viruses was greater than that for bacteria; and among the positive specimens for viruses, the positive rate for norovirus was higher than that for rotavirus (18). Norovirus has a heavy disease burden in Jiangsu Province. Currently, researchers in Jiangsu Province have made significant progress in studying the molecular epidemiological characteristics of norovirus outbreaks (19–22). However, epidemiological characterization of large sample sizes is still limited for a large number of reported norovirus outbreaks in Jiangsu Province. Previous epidemiological studies on norovirus outbreaks usually calculated the total attack rate (*TAR*) or duration of the outbreak as the dependent variable and collected risk factors associated with the infection as independent variables for statistical analysis, and finally found the epidemiological characteristics of norovirus outbreaks and factors influencing the severity of outbreaks in a certain area during a certain period of time, providing a scientific basis for controlling norovirus outbreaks (23–25). However, we believe that studies on the *TAR* do not completely reflect the transmissibility of the virus, and it is necessary to construct mathematical models to quantify the transmissibility of norovirus in outbreaks. Current mathematical models used to calculate the transmissibility of infectious diseases include agent-based models and ordinary differential equation models. In our previous study, the transmissibility of norovirus in interpersonal transmission and water transmission modes was described by constructing ordinary differential equation models (26). Therefore, in order to identify as many risk factors as possible and determine the epidemiological characteristics of norovirus outbreaks in Jiangsu province, we collected all outbreak investigation reports from 2012 to 2018 in Jiangsu Province, sequenced all positive specimens eligible for sequencing to determine the genotype, determined the *TAR* of each outbreak, and constructed transmission dynamics models for different transmission routes to calculate the transmissibility, and performed statistical analyses of risk factors associated with the *TAR* and transmissibility.

MATERIALS AND METHODS

Research Design

Our study is an observational study that combines fieldwork and transmissibility. This study consists of three parts, the logical relationship of which is shown in **Figure 1**. According to the investigation report, we established a database, numbered each outbreak, and transformed the description of the outbreak into tabular data. We could find out epidemiological data, demographic data and influencing factors from investigation reports. In order to identify genotypes, we also collected and detected samples. From the report, we could know exactly the transmission route of each outbreak, and select different transmission models according to different transmission routes. The independent variable was the data extracted from each investigation report; the dependent variables were the *TAR* and reproduction number (R_0). The *TAR* was calculated according to the outbreak data, and R_0 was calculated using the transmission dynamics model. In this study, prevalence was used for model fitting. The transmission dynamics model aims to refine the transmission process of infectious diseases in the whole population, so as to explain the transmission mechanism of infectious diseases. According to the influencing factors in the report, we explored whether each influencing factor would affect the *TAR* and R_0. Chi-square analysis was used to compare the rates of multiple samples, and analysis of variance was used to compare the mean of multiple samples.

Data Sources

Database

The outbreak database (**Supplementary Table 1**) contained the city where the outbreak occurred, the number of people affected by the outbreak (susceptible people), the number of cases, the number of deaths, the location type, the onset date of the initial case, the transmission route and genotype. The data source of the outbreak database was mainly based on investigation reports. Contents of the report mainly included the investigation process and contents of the aggregated epidemic and outbreak, mainly including the basic situation investigation of the epidemic occurrence institution, on-site epidemiological investigation, hygienic investigation and epidemic termination evaluation, as well as the sample collection and laboratory test results involved in the investigation. All contents of the investigation report were specified in the "Guidelines on outbreak investigation, prevention and control of norovirus infection" issued by China Center for Disease Control and Prevention (27).

Case definition:

(1) Suspected case: That is, acute gastroenteritis cases, defined as those who defecate $\geq$ 3 times within 24 h and have character changes (dilute watery stool), and/or vomit $\geq$ 2 times within 24 h. (2) Clinical diagnostic cases: In the cluster epidemic or outbreak caused by norovirus infection, the cases that meet the definition of suspected cases and are epidemiologically related to laboratory diagnosed cases. (3) Laboratory diagnosed cases: In suspected cases or clinical diagnostic cases, stool, anal swab or vomit samples are positive for norovirus nucleic acid or ELISA antigen.

Judgment criteria of cluster epidemic and outbreak:

(2) Cluster epidemic: Within 3 days, 5 or more epidemiologically linked cases of norovirus infection occur in the same school, childcare institution, medical institution, nursing home, factory, construction site, cruise ship, community or village, and other crowded places or sites, of which at least 2 are laboratory diagnosed cases. (2) Outbreak: 20 or more epidemiologically linked cases of norovirus infection in the same school, childcare institution, medical institution, nursing home, factory, construction site, cruise ship, community or village, and other crowded places or sites within 7 days, of which at least 2 are laboratory diagnosed cases. (3) If no laboratory testing capability for norovirus is available or in the early stage of outbreak detection, a suspected outbreak or outbreak of norovirus infection can be determined if the following four characteristics of Kaplan Criteria are met: (1) more than half of the patients present vomiting symptoms; (2) average incubation period of 24–48 h; (3) average duration of illness 12–60 h; (4) exclude bacterial, parasitic and other pathogenic infections. The sensitivity of the Kaplan Criteria for identifying norovirus outbreaks is 68% and the specificity is 99%.

Laboratory Detection

All stool specimens collected from outbreaks would be tested for norovirus. The stool suspensions were diluted 10-fold with saline solution. Viral nucleic acids were extracted and tested using the MagMAXTM-96 Viral RNA Isolation Kit (Applied Biosystems, Foster City, CA) and the Qiagen Probe reverse-transcriptase polymerase chain reaction (RT-PCR) Kit (Qiagen, Hilden, Germany) on a 7500 real-time PCR platform (Applied Biosystems, Foster City, CA) with primers as described (28).

Genotyping and Phylogenetic Analysis

In our study, norovirus nucleic acid was detected in samples collected from each outbreak. All norovirus-positive samples were detected with a region of 543 bp (GI) or 557 bp (GII) targeted at the ORF1/ORF2 junction of the viral genome by one-step RT-PCR for norovirus genotyping (29). If sequencing failed, the VP1 gene in ORF2 was amplified using the previously described primers G1SKF/G1SKR(GI) or G2SKF/G2SKR(GII) (30). However, the CT values of some samples were >33, the viral load was low, and sufficient amplification products could not be obtained, meaning that sequencing conditions were not met and sequencing failed. The genotypes were determined using the norovirus automated genotyping tool (http://www.rivm.nl/mpf/norovirus/typing tool) and the human calicivirus typing tool (http://norovirus.ng.philab.cdc.gov). We selected 25 representative gene sequences for gene polymorphism demonstration, which covered all genotypes of norovirus causing norovirus outbreaks in Jiangsu Province from 2012 to 2018. Phylogenetic trees were constructed using the maximum likelihood algorithm with 1,000 bootstrap replicates and a Kimura2-parameter model in MEGA 7.0 (31); the norovirus reference sequences were obtained from the GenBank database. Nucleotide sequences obtained from this study were deposited in GenBank under the accession numbers MZ373204-MZ373210 and MZ373215-MZ373233.

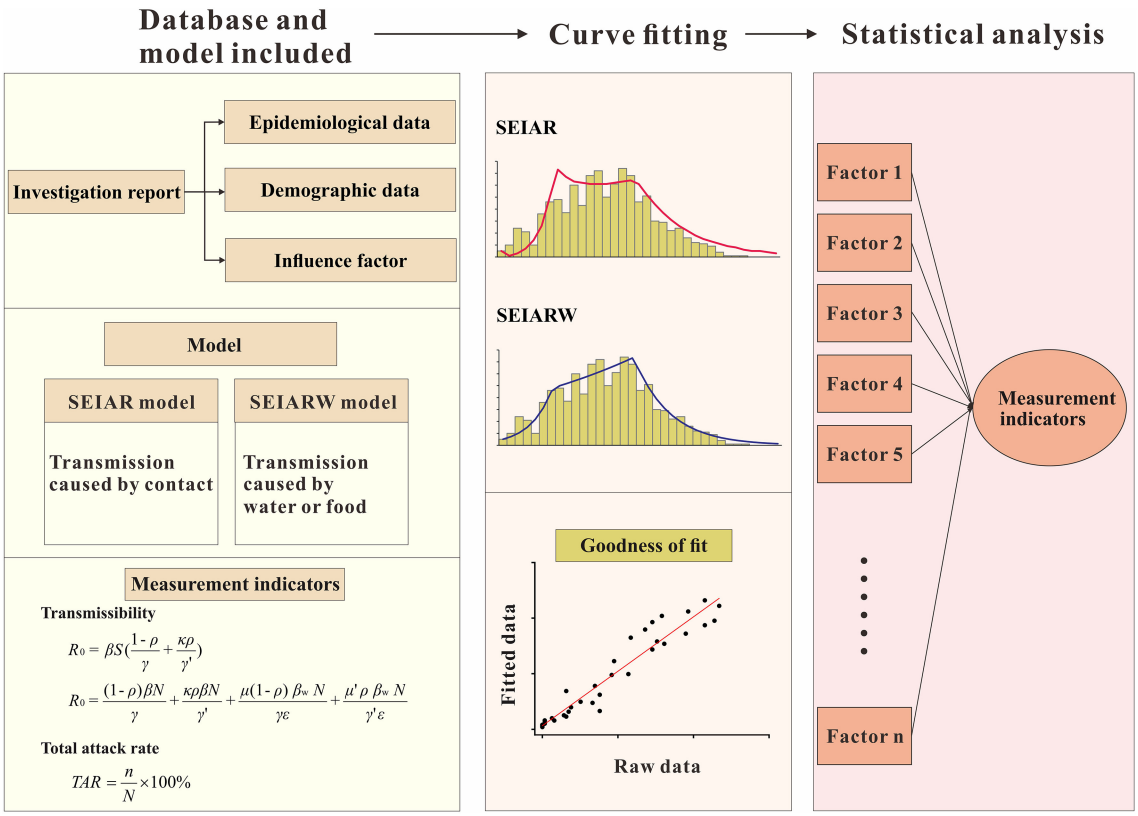

FIGURE 1 | Flow chart of norovirus outbreak research in Jiangsu Province.

Transmission Model

Similar to a previous study (32), a susceptible-exposed-symptomatic-asymptomatic-recovered (SEIAR) and a susceptible-exposed-symptomatic-asymptomatic-recovered-transmission media (SEIARW) model was employed for the simulation. The SEIAR model of interpersonal transmission and SEIARW model of water or food transmission were used to assess the transmissibility of norovirus in each outbreak. In this study, Berkeley Madonna 8.3.18 (developed by Robert Macey and George Oster of the University of California at Berkeley) was used for curve fitting and simulation, and SPSS 21.0 (IBM Company, U.S.A.) was used to calculate the coefficient of determination (R^2) and P-value.

Interpersonal Transmission

In the SEIAR model, the population was divided into five groups: susceptible (S), exposed (E), symptomatic (I), asymptomatic (A), and recovered (R)

The assumptions of the model are as follows:

A susceptible person becomes the exposed person after coming into contact with the symptomatic and asymptomatic infected person at the speed of βSI and $\beta S\kappa A$. Where β represents the probability of transmission per contact, and κ represents the transmissibility of asymptomatic cases compared to symptomatic cases.

After the incubation period ($1/\omega$) and latent period ($1/\omega'$), the exposed persons become symptomatic or asymptomatic; where ρ represents the proportion of asymptomatic infections.

Symptomatic and asymptomatic cases recovered after a period of $1/\gamma$ and $1/\gamma'$.

The flowchart of the model is shown in **Figure 2A**.

The equations of the model are as follow:

$$\begin{aligned}
\frac{dS}{dt} &= -\beta S\left(I + \kappa A\right) \\
\frac{dE}{dt} &= \beta S\left(I + \kappa A\right) - \left(1-\rho\right)\omega E - \rho\omega' E \\
\frac{dI}{dt} &= \left(1-\rho\right)\omega E - \gamma I \\
\frac{dA}{dt} &= \rho\omega' E - \gamma' A \\
\frac{dR}{dt} &= \gamma I + \gamma' A
\end{aligned}$$

Water or Food Transmission

In the SEIARW model, the water or food transmission medium (W) is added.

The assumptions of the model are as follows:

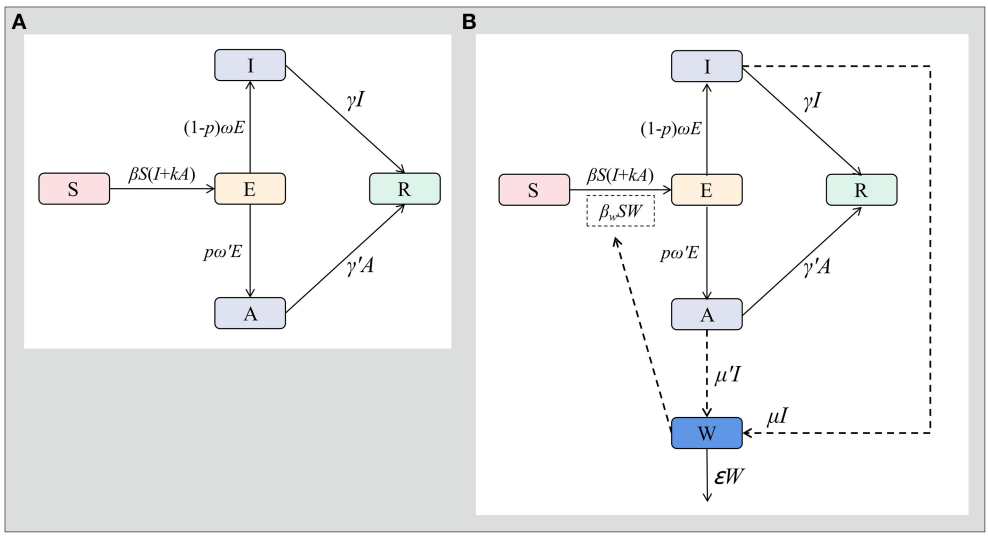

FIGURE 2 | SEIAR model and SEIARW model for norovirus transmission in Jiangsu Province. [**A**: susceptible-exposed-symptomatic-asymptomatic-recovered model (SEIAR model), **B**: susceptible-exposed-symptomatic-asymptomatic-recovered-transmission media model (SEIARW model)].

Both symptomatic and asymptomatic cases contaminate water or food by shedding pathogens into compartment W at shedding rates of μI and μ'A. μ and μ' represent shedding coefficients. When exposed to contaminated water or food, the susceptible people will become exposed people at the rate of $\beta_w SW$, which represents the coefficient of water or food transmission.
The flowchart of the model is shown in **Figure 2B**.
The equations of the model are as follow:

$$\begin{aligned}
\frac{dS}{dt} &= -\beta S\left(I+\kappa A\right)-\beta_w SW \\
\frac{dE}{dt} &= \beta S\left(I+\kappa A\right)+\beta_w SW-\left(1-\rho\right)\omega E-\rho\omega' E \\
\frac{dI}{dt} &= \left(1-\rho\right)\omega E-\gamma I \\
\frac{dA}{dt} &= \rho\omega' E-\gamma' A \\
\frac{dR}{dt} &= \gamma I+\gamma' A \\
\frac{dW}{dt} &= \varepsilon(I+ca-w)
\end{aligned}$$

Parameter Source

Model 1 included seven parameters: β, k, ω, ω', ρ, γ and γ' (**Table 1**). β was adjusted using the actual outbreak data and model. Previous researches have shown that the incubation period of norovirus was 12–48 h (33, 34), therefore, our model chose 1 d as the incubation period ($\omega = 1$). A study showed that norovirus started to shed within an average of 36 h (range: 18–110) after infection, and the virus could be discharged for approximately 26 d (range: 11–54) (35), thus, the latent and infectious period was 1 d and 26 d, so ω'=1, $\gamma' = 0.03846$. The course of norovirus infectious diarrhea is generally 1–5 d (36, 37), with some cases reaching 4–6 d (38, 39). According to the Kaplan principle of norovirus infectious diarrhea diagnosis commonly used in the United States (40), the average course of the disease is 1–3 d. In this study, 3 d were used for the modeling study, namely $\gamma = 0.3333$. The asymptomatic infection ratio of this disease potentially reaches 30% (35, 41, 42); therefore, $\rho = 0.3$ was used.

Model 2 included 14 parameters. The normalized 10 parameters were the parameters that required specific values, which were b, b_w, κ, ω, ω', ρ, γ, γ', c, and ε. Among them, b and $b_w$were fitted using the actual outbreak data and model.

The shedding rate of the asymptomatic compared to the infectious was c, and $\mu' = c/\mu$ (43); subsequently, the specific value of the parameter was calculated by fitting the actual epidemic data. The survival time of norovirus in the external environment was approximately about 7–12 d (44–46), and the longest was 21–28 d (47–52). In this study, 10 d were used for the study; therefore, $\varepsilon = 0.1$ was used.

Evaluation Index

In this study, R_0 was used to evaluate the transmissibility of norovirus. In Model 1, the R_0 formula is expressed as follows:

$$R_0 = \beta S\left(\frac{1-\rho}{\gamma}+\frac{\kappa\rho}{\gamma'}\right)$$

In Model 2, R_0 formula is expressed as follows:

$$R_0 = \frac{\left(1-\rho\right)\beta N}{\gamma}+\frac{\kappa\rho\beta N}{\gamma'}+\frac{\mu\left(1-\rho\right)\beta_w N}{\gamma\varepsilon}+\frac{\mu'\rho\beta_w N}{\gamma'\varepsilon}$$

R_0 was divided into two parts (R_{unc} and R_{con}) where R_{unc} and R_{con} represent the uncontrolled and controlled R_0, respectively. The epidemic curve was divided into two stages based on the increase and decrease. The increasing stage of the epidemic curve indicates that the intervention measures are yet to exert their effects; therefore, it is a better representation of the real

TABLE 1 | Parameter definitions and values.

Parameter	Description	Unit	Value	Method
β	Person–to-person contact rate	$km^2 \cdot individuals^{-1} \cdot day^{-1}$	–	Curve fitting
β_w	Reservoir– to-person contact rate	$mL^3 \cdot cells^{-1} \cdot day^{-1}$	–	Curve fitting
K	Relative transmissibility rate of asymptomatic to symptomatic individuals	1	–	Curve fitting
Ω	Relative incubation rate*	day^{-1}	1	(33, 34)
ω'	Relative latency rate*	day^{-1}	1	(35)
P	Asymptomatic infection ratio	1	0.3	(35, 41, 42)
Γ	Recovery rate of the infected	day^{-1}	0.3333	(36–40)
γ'	Recovery rate of the asymptomatic	day^{-1}	0.03846	(35)
?	Pathogen lifetime relative rate	day^{-1}	0.1	(44–52)
?	Person–to-reservoir contact rate ("shedding" by Infectious)	$cells \cdot mL^{-3} \cdot day^{-1} \cdot km^2 \cdot individuals^{-1}$	–	c/μ' (43)
μ'	Person–to-reservoir contact rate ("shedding" by Asymptomatic)	$cells \cdot mL^{-3} \cdot day^{-1} \cdot km^2 \cdot individuals\text{-}1$	–	c/μ (43)
C	Shedding rate of the asymptomatic comparing to the infectious	1	–	Curve fitting

**Incubation period is the time elapsed between infected and symptoms are first apparent, and latent period means the time from infected to infectiousness, in this table, incubation period = 1/ω, latent period = 1/ω′.*

transmissibility of the disease. Therefore, we used the data for epidemiological analysis and recorded the data as R_{unc}.

TAR was used to evaluate the effects of various preventive and control measures. where *n* is the cumulative number of cases and *N* is the number of susceptible people in an outbreak.

The formula of *TAR* is:

$$TAR = \frac{n}{N} \times 100\%$$

Statistical Method

All statistical analyses were performed using SPSS 21.0 (IBM Company, U.S.A.). For *TAR*, the chi-square test was used for comparison between the groups. Groups with significant differences were analyzed using the Bonferroni method for further pairwise comparisons. Statistical significance was set at $P < 0.05$ for both between group and pairwise comparisons. For R_0, a homogeneity test of variance was first performed. If the variance was homogeneous, analysis of variance was used for inter-group comparison. Simultaneously, the LSD method was used for further pairwise comparison. If the homogeneity of variance was not satisfied, the Kruskal Wallis rank-sum test was used for inter-group comparison.

RESULT

Epidemiological Characteristics

From 2012 to 2018, data from 206 norovirus outbreaks were collected in Jiangsu Province; all were able to calculate for the *TAR*. The epidemic curves of 206 outbreaks are showed in **Figure 3**. However, only 145 met the conditions for calculating transmissibility, and the basic reproduction number was calculated. The fitting effects of 145 outbreaks are shown in **Supplementary Figure 1** which found the mean R^2 was 0.76 and all *P*-values were significant.

For risk factors for *TAR* and transmissibility, we have the following findings (**Tables 2**, **3**).

The mean *TAR* of 206 outbreaks was 2.6% and the mean R_{unc} of 145 outbreaks was 12.2 (95% confidence interval: 10.7–13.6) from 2012 to 2018.

The map showed significantly more outbreaks in southern Jiangsu compared to northern Jiangsu; furthermore, the *TAR* and R_{unc} showed the highest trend in southern Jiangsu (**Figure 4**). Wuxi City reported the most outbreaks (65 outbreaks), and Xuzhou City reported the least outbreaks (two outbreaks). There was a significant difference in mean *TAR* between cities ($\chi^2 =$ 1,458.876, $P < 0.05$). The highest and lowest mean *TAR* were found in Suzhou City (4.7%) and Suqian City (0.4%), respectively. There was no significant difference in transmissibility between cities. Regarding location, the *TAR* between different cities was statistically different ($\chi^2 = 193.783$, $P < 0.05$), the highest and lowest mean *TAR* were observed in central and northern Jiangsu, respectively.

The number of outbreaks showed a significant upward trend from 2012 to 2018. A big turning point was observed in 2016–2017, with 14 and 78 outbreaks reported, respectively. There was a significant difference in mean *TAR* between the years analyzed ($\chi^2 = 998.138$, $P < 0.05$). Chi-square test pairwise comparison results showed differences between all years except 2013 and 2017, and 2014 and 2016. The mean *TAR* and R_{unc} tortuously changed over time, with a peak every 3 years.

In the four seasons, the number of outbreaks in summer was the lowest (six outbreaks); more outbreaks were observed in autumn and winter, 71 and 70, respectively. There was a significant difference in the mean *TAR* between seasons ($\chi^2 = 237.62$, $P < 0.05$). The summer had the lowest mean *TAR*. The mean *TAR* was found to be high from February to March and October (3.7, 3.7, 2.9%, respectively). The season significantly affected the *TAR* in all seasons except between autumn and winter. There was no significant difference in transmissibility between seasons. Outbreaks with high R_{unc} were concentrated in February, November, and April (17.9, 12.8, and 12.6, respectively).

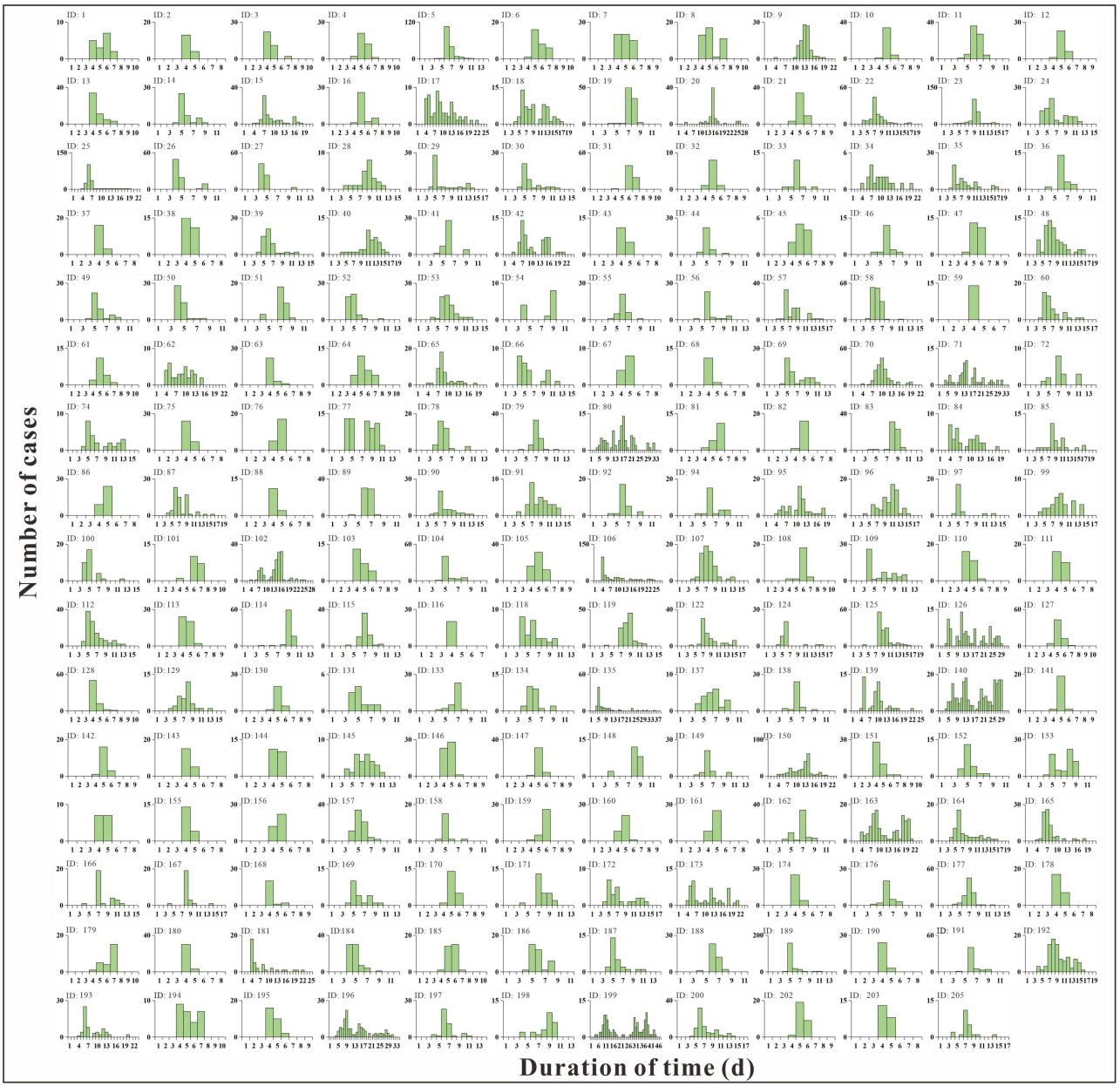

FIGURE 3 | Epidemic curve of 206 outbreaks.

Most outbreaks occurred in urban areas (160 outbreaks) compared to rural areas (46 outbreaks). The mean *TAR* between rural and urban areas was significantly different ($\chi^2 = 502.826$, $P < 0.05$). The mean *TAR* in rural areas was higher compared to urban areas (3.9 and 2.4%, respectively). The mean R_{unc} in rural areas and urban areas were 11.4 and 12.4, respectively.

Among 206 outbreaks, 196 occurred in schools, and ten occurred in places other than schools. There was a significant difference in the mean *TAR* between the categories of places analyzed ($\chi^2 = 1{,}565.869$, $P < 0.05$). Regarding the type of location, no significant difference was observed between common colleges and secondary vocational schools and 9-year school and 12-year school; however, other places significantly affected the mean *TAR*. The mean *TAR* in non-school places was 8.4%. Among the schools, kindergartens had the highest mean *TAR* (5.7%). There was no significant difference in transmissibility between the categories of places analyzed. Among the outbreak sites, the mean R_{unc} for kindergartens was 16.1, the mean R_{unc} observed in common colleges and secondary vocational schools was 6.5. Furthermore, the mean R_{unc} of non-schools was only 7.6.

Most of the outbreaks occurred in places with a small number of susceptible people, 61 and 70 outbreaks occurred in places with a number of <1,000 and 1,000–2,000 susceptible people,

TABLE 2 | TAR and transmissibility and influence factors in 206 outbreaks of norovirus in Jiangsu Province.

	TAR (%)			R_{unc}		
	N	**Mean**	**95%CI**	***N***	**Mean**	**95%CI**
Influence factors	206	2.6		145	12.2	10.7–13.6
Year						
2012	4	1	0.9–1.2	3	11.3	1.2–21.4
2013	6	3.2	2.9–3.6	5	12.3	4.5–20.1
2014	15	1.6	1.5–1.7	9	10.4	5.9–15
2015	22	2	1.9–2.2	16	12.7	7.6–17.7
2016	14	1.5	1.4–1.6	11	11.9	7.3–16.4
2017	78	3.5	3.5–3.6	57	12.6	9.9–15.4
2018	67	2.5	2.4–2.6	44	11.9	9.5–14.3
Month						
1	8	2.2	1.9–2.4	5	8.1	0.4–15.8
2	27	3.7	3.5–3.9	20	17.9	11.7–24.2
3	37	3.7	3.5–3.8	32	10.4	7.2–13.5
4	17	3.1	2.9–3.3	11	12.6	7.9–17.4
5	5	1.5	1.3–1.6	4	7.8	4.4–11.2
6	6	1.4	1.1–1.6	2	10.4	0–39.7
7	0	NA	NA	0	NA	NA
8	0	NA	NA	0	NA	NA
9	7	2.4	2.2–2.6	5	7.1	1.7–12.5
10	25	2.9	2.7–3.1	15	12.4	9.2–15.6
11	39	2.4	2.3–2.5	25	12.8	9.5–16.2
12	35	1.8	1.7–1.9	26	11.6	8.6–14.6
Seasons						
Spring	59	3.2	3.1–3.3	47	10.7	8.3–13
Summer	6	1.4	1.1–1.6	2	10.4	0–39.7
Autumn	71	2.5	2.4–2.6	45	12.1	9.9–14.2
Winter	70	2.4	2.3–2.5	51	13.7	10.8–16.7
City						
Changzhou	46	3	2.9–3.1	37	10.5	8.2–12.8
Huai'an	3	1.4	1.1–1.7	3	10.5	0–26.2
Lianyungang	2	3.1	2.2–3.9	1	12.7	NA
Nanjing	20	2.4	2.3–2.5	15	17.3	12.0–22.5
Nantong	4	2.7	2.1–3.3	2	28.5	0–81.7
Suzhou	17	4.7	4.4–5	11	17.4	8.0–26.7
Taizhou	13	1.4	1.3–1.5	4	13.7	0–33.5
Wuxi	65	2.2	2.2–2.3	47	10.8	8.5–13
Suqian	3	0.4	0.3–0.5	3	8.1	1.9–14.3
Xuzhou	2	3.5	3.1–3.9	1	9.0	NA
Yancheng	6	1.9	1.7–2.1	5	8.6	0–18.4
Yangzhou	18	4.5	4.3–4.8	13	11.0	6.6–15.5
Zhenjiang	7	2.2	2–2.5	3	15.2	0–47.4
Region						
South of Jiangsu	155	2.7	2.6–2.7	113	12.3	10.6–14
Middle of Jiangsu	35	2.9	2.8–3.1	19	13.4	8.8–18.1
North of Jiangsu	16	1.7	1.6–1.8	13	9.3	5.9–12.6
Rural and Urban						
Rural	46	3.9	3.7–4	25	11.4	9.3–18.3
Urban	160	2.4	2.3–2.4	120	12.4	10.3–13.3

(Continued)

TABLE 2 | Continued

	TAR (%)			R_{unc}		
	N	**Mean**	**95%CI**	***N***	**Mean**	**95%CI**
Categories of places						
Non-school places	10	8.4	7.5–9.3	8	7.6	2.9–12.3
Kindergarten	26	5.7	5.3–6.1	16	16.1	10.8–21.4
Primary school	108	2.4	2.3–2.5	73	13.5	11.4–15.5
Middle school	39	3.4	3.3–3.5	32	10.6	7.8–13.3
Common Colleges and Secondary vocational school	14	1.7	1.6–1.8	11	6.5	3.0–10
Nine-year school and Twelve-year school	9	1.7	1.5–1.9	5	10.9	0–28.9
Population						
0–999	61	5.6	5.3–5.8	39	13.4	10.3–16.5
1000–1999	70	3.3	3.2–3.4	53	12.8	10.4–15.1
2000–2999	45	2.6	2.5–2.7	35	11.2	8.2–14.1
≥3000	30	1.3	1.3–1.4	18	9.7	5.9–13.5
Route of transmission						
Water or food to person	8	2.2	2–2.3	7	14.0	5.3–22.7
Person to person	198	2.6	2.6–2.7	138	12.1	10.6–13.6
Virus group						
GI	9	1.3	1.1–1.4	7	10.3	4.1–16.6
GII	132	2.7	2.7–2.8	98	12.8	11.0–14.6
Virus genotype						
GII.2 [P16]	87	3.1	3–3.2	64	13.3	10.9–15.7
GII.17 [P17]	21	2.2	2–2.3	16	10.8	7.6–14.1
GII.3 [P12]	11	2	1.8–2.2	7	17.4	6.1–28.7
GII.4 [P31]	6	1.5	1.3–1.6	5	8.0	0–16.2
GI.6*	3	1.8	1.4–2.1	2	6.6	0–21.7
GII.6 [P7]	3	3.8	3.1–4.6	3	15.6	9–22.1
GI.2*	2	2.8	2.3–3.3	2	16.7	0–114.7
GI.2 [P2]	2	1.8	1.3–2.2	2	9.3	0–61.8
GI.3 [P13]	1	2.8	NA	NA	NA	NA
GI.6 [P11]	1	0.2	NA	1	7.2	NA
GII.1*	1	2.4	NA	1	14.5	NA
GII.13 [P16]	1	4.6	NA	1	3.4	NA
GII.14 [P7]	1	3.3	NA	1	4.5	NA
GII.2 [P2]	1	3.4	NA	NA	NA	NA

**RdRp Genotype not detected.*

respectively, and the least outbreaks occurred in places with a small number of susceptible people, only 30. A comparison of the different numbers of susceptible people regarding the mean *TAR* showed statistically significant differences ($\chi^2 = 22.19$, $P < 0.05$). The highest and lowest *TAR* means were found in places with <1,000 people (5.6%) and places with more than 3,000 people (1.3%), respectively. Further, there was no significant difference in transmissibility between places with differing numbers of susceptible people, the mean R_{unc} of places with <1,000 people was 13.4, and the mean R_{unc} of places with more than 3,000 people was 9.7.

Regarding the transmission routes, 198 outbreaks were interpersonal transmission and eight were waterborne or foodborne transmission. The mean *TAR* was significantly different between different transmission routes ($\chi^2 = 2{,}296.419$, $P < 0.05$). The mean *TAR* of waterborne or foodborne transmission was lower compared to interpersonal transmission (2.2 and 2.6%, respectively). There was no significant difference in transmissibility between transmission routes. The mean R_{unc} of water or food transmission route and human transmission route was 14.0 and 12.1.

Genotype sequencing was completed in 141 outbreaks; among which, the GII genotype caused the highest number of outbreaks (132/141). Among the GII genotypes, the GII.2 [P16] genotype caused the most outbreaks (87 outbreaks). As shown in **Figure 5**, the outbreaks in 2012–2014 were mainly caused by the GII.4

TABLE 3 | Chi-square test and pairwise comparison of the differences in TAR of influencing factors.

Classification	Variables	Cases	Non-case	χ^2	*P*-value
Year	2012[a]	147	14,176	998.138	$P < 0.05$
	2013[b]	326	9,715		
	2014[c]	545	33,250		
	2015[d]	883	42,322		
	2016[c]	513	34,179		
	2017[b]	4,784	129,986		
	2018[e]	3,108	122,171		
Month	1[a,b]	391	17,783	925.107	$P < 0.05$
	2[c]	1,458	37,600		
	3[c]	2,268	59,199		
	4[d]	772	23,968		
	5[e]	263	17,663		
	6[e]	155	11,286		
	7				
	8				
	9[b]	494	20,324		
	10[d]	909	30,539		
	11[b]	1,961	79,623		
	12[a]	1,635	87,814		
Seasons	Spring[a]	3,303	100,830	237.62	$P < 0.05$
	Summer[b]	155	11,286		
	Autumn[c]	3,364	130,486		
	Winter[c]	3,484	143,197		
City	Changzhou[a]	2,679	87,170	1,458.876	$P < 0.05$
	Huai'an[b,c]	94	6,727		
	Lianyungang[a,d,e,f,g,h,i,j,k,l,m,n,o,p,q]	49	1,555		
	Nanjing[n,o,p,q]	1,080	44,012		
	Nantong[a,j,k,l,m,p,q]	80	2,844		
	Suzhou[i]	987	19,906		
	Taizhou[c]	414	29,444		
	Wuxi[g,h,l,m,o,q]	2,563	111,413		
	Suqian[r]	58	13,904		
	Xuzhou[a]	246	6,804		
	Yancheng[b,f,h,k,m]	334	16,926		
	Yangzhou[e,i]	1,334	28,114		
	Zhenjiang[d,f,g,h,j,k,l,m,n,o,p,q]	388	16,980		
Region	North of Jiangsu[a]	7,697	279,481	193.783	$P < 0.05$
	Middle of Jiangsu[b]	1,828	60,402		
	South of Jiangsu[c]	781	45,916		
Rural and Urban	Rural[a]	2,492	61,506	502.826	$P < 0.05$
	Urban[b]	7,814	324,293		
Categories of places	Non-school places[a]	320	3,477	1,565.869	$P < 0.05$
	Kindergarten[b]	767	12,661		
	Primary school[c]	4,958	202,153		
	Middle school[d]	2,706	76,743		
	Common Colleges and Secondary vocational school[e]	1,112	65,204		
	9-year school and 12-year school[e]	443	25,561		
Population	0–999[a]	1,929	32,570	22.19	$P < 0.05$
	1,000–1,999[b]	3,580	106,354		

(Continued)

TABLE 3 | Continued

Classification	Variables	Cases	Non-case	χ^2	*P*-value
	2,000–2,999[c]	2,890	106,526		
	≥3,000[d]	1,907	140,349		
Route of transmission	Water or food to person[b]	627	28,182	2,296.419	$P < 0.05$
	Person to person[a]	9,679	357,617		
Virus group	GI[a]	353	27,758	220.681	$P < 0.05$
	GII[b]	6,921	245,709		
Virus genotype	GII.2 [P16][a]	5,082	160,556	677.818	$P < 0.05$
	GII.17 [P17][b,c,d,e]	835	37,256		
	GII.3 [P12][d,e]	402	20,094		
	GII.4 [P31][f]	295	19,690		
	GI.6[c,e,f]	106	5,914		
	GII.6 [P7][a,g]	99	2,487		
	GI.2[a,b]	133	4,579		
	GI.2 [P2][b,c,d,e,f]	66	3,675		
	GI.3 [P13][a,b,c,d,e,f,g]	18	620		
	GI.6 [P11][h]	30	12,970		
	GII.1[a,b,c,d,e,f]	45	1,856		
	GII.13 [P16][g]	117	2,438		
	GII.14 [P7][a,b,c,d,e,g]	33	959		
	GII.2 [P2][a,b,c,d,e,f,g]	13	373		

The same letter of the mark indicates that there is no difference between the two groups of data, and the different letter indicates that the difference is statistically significant. For example, the mean TAR in spring was statistically different from all other seasons; the mean TAR in summer was statistically different from all other seasons; the mean TAR in autumn was statistically different from spring and summer only, but not from winter; and the mean TAR in winter was statistically different from spring and summer only, but not from autumn.

[P31] and GII.P17 genotypes, and the majority of outbreaks in 2015–2016 were caused by the GII.17 [P17] genotype, while a change occurred after 2016 when the GII.2 [P16] genotype became dominant. In 2017 and 2018, outbreaks caused by the GII.2 [P16] genotype accounted for 91.0% (50/55) and 73.5% (36/49) of all outbreaks in that year, respectively. The GII.13 [P16] genotype had the highest mean *TAR* (4.6%), while GI.6 [P11] had the lowest (0.2%). Furthermore, the mean R_{unc} of GII.3 [P12] was 17.4, and the mean R_{unc} of GII.13 [P16] was 3.4.

Genetic Diversity

To show the genetic diversity of norovirus in Jiangsu Province, 25 norovirus sequences with representative genotypes were analyzed using MEGA 7.0 software. Phylogenetic trees were constructed based on the partial RdRp gene (230 bp) and VP1 gene (280 bp) using the maximum likelihood method. As shown in **Supplementary Figure 2**, eight genotypes had discordant capsid and polymerase genotypes and were considered intergenotype recombinant strains (GI.6 [P11], GII.2 [P16], GII.3 [P12], GII.4 Sydney [P31], GII.6 [P7], GII.13 [P16], GI.3 [P13], and GII.14 [P7]), and three genotypes had accordant capsid and polymerase genotypes (GI.2 [P2], GII.2 [P2], and GII.17 [P17]).

DISCUSSION

In this study, we used *TAR* and a transmission dynamics model to explore which factors would influence the norovirus outbreaks in Jiangsu Province.

Regarding time, the number of reported cases increased sharply in 2017 and 2018. Jiangsu Province began to establish a norovirus surveillance system in 2012, using a unified case definition, outbreak determination principles, and laboratory testing methods for surveillance, so we consider our description that the number of norovirus outbreaks is increasing is consistent with the real epidemiologic characteristics. Meanwhile, we observed that the GII.2 [P16] genotype caused more than half of the norovirus outbreaks in Jiangsu province in 2017 and 2018. Previously, a study on norovirus outbreak surveillance in China had already found that the number of norovirus outbreaks increased substantially at the end of 2016, greatly exceeding the number reported in the same month in the previous 4 years, and the majority of these outbreaks were associated with GII.2 [P16] (53). The researchers analyzed this finding and concluded that the GII.2 [P16] genotype evolved extremely fast, which most likely led to a sudden increase of this virus (54). We, therefore, considered that the prevalence of the GII.2 [P16] genotype is responsible for the surge in the number of norovirus outbreaks in Jiangsu Province between 2017 and 2018.

Regarding the season, in our study, *TAR* was significantly lower during summer, and norovirus outbreaks in Jiangsu Province mainly occurred in the colder weather in spring, autumn, and winter, which is consistent with the results of previous studies (55, 56). Regarding the regional distribution, the increased outbreaks in southern Jiangsu despite the higher *TAR* in central Jiangsu may be attributed to the increased

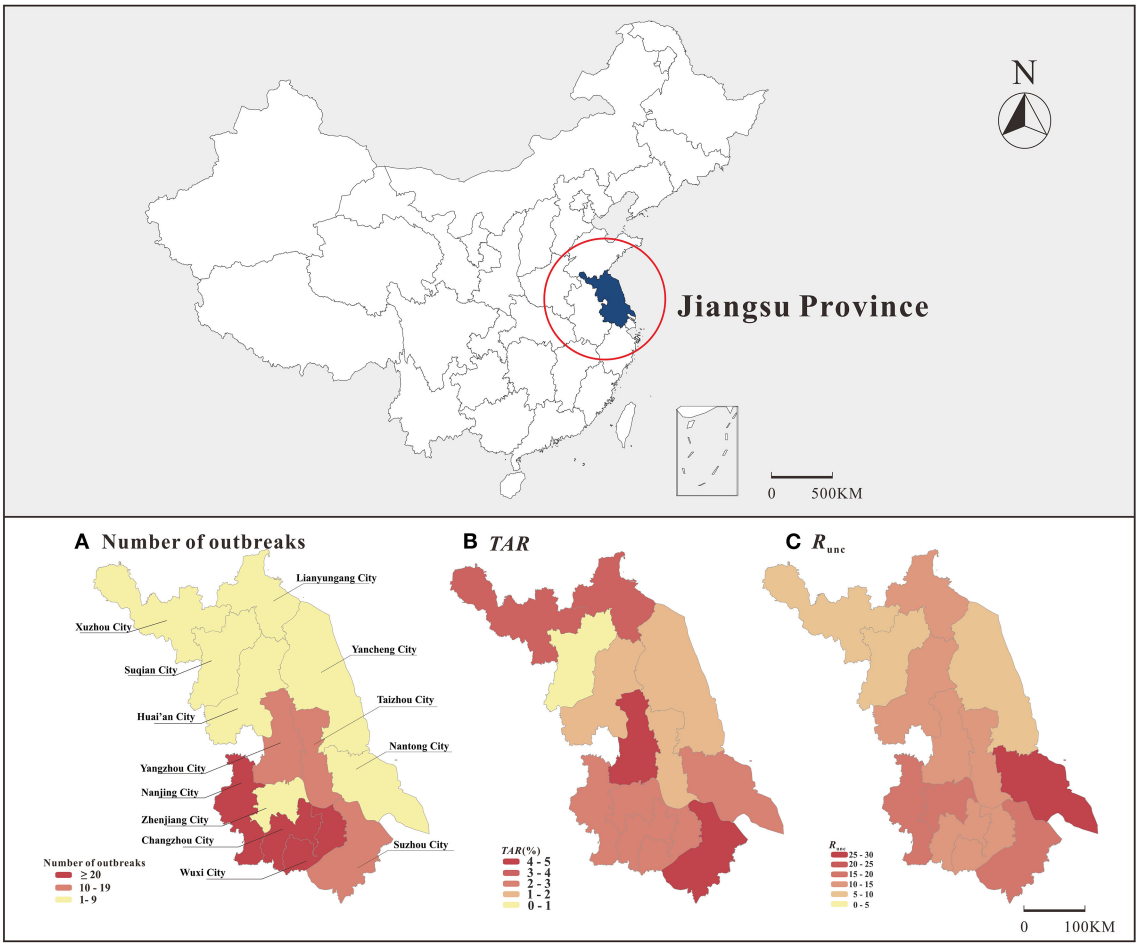

FIGURE 4 | Distribution of number of outbreaks, *TAR* and transmissibility on map of Jiangsu Province (**A**: number of outbreaks, **B**: *TAR*, **C**: R_{unc}).

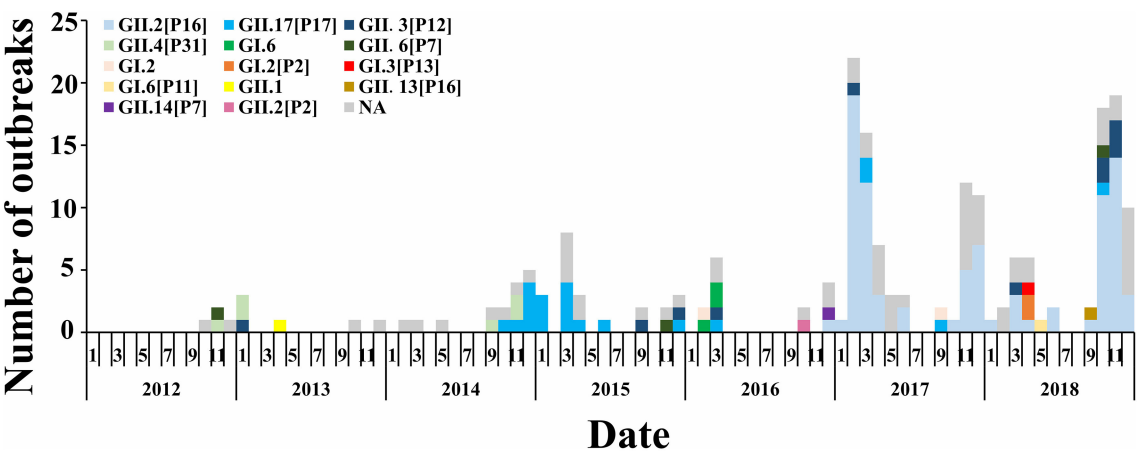

FIGURE 5 | Changes of norovirus genotypes in Jiangsu Province from 2012 to 2018.

sensitivity to disease surveillance and more timely detection and reporting of the outbreaks in southern Jiangsu. Regarding rural and urban sites, the mean *TAR* in rural areas was higher compared to urban areas, which may be related to poor sanitation in rural areas and the untimely adoption of control measures.

Consistent with previous studies (8–13), the outbreaks we monitored occurred more often in schools than in hospitals and long-term care facilities, this phenomenon is largely due to the fact that: (1) In China (56), schools have a more standard outbreak screening system in which the government requires schools to check and screen students for symptoms of fever, vomiting, or diarrhea every morning. In contrast, hospitals or long-term care facilities do not see similar mechanisms for disease detection and reporting. (2) Long-term care facilities are important health care settings outside China. In the United States (57), long-term care facilities serve more than 2 million Americans each year, and local health units often provide infection control courses to facility staff. Ontario requires by law that each health unit must provide in-service training to long-term care facilities at least once a year. In France (58), epidemiological surveillance of acute gastroenteritis outbreaks in various types of nursing homes has been conducted since November 2010. Well-established facilities and systematic services make such settings capable of detecting and reporting norovirus outbreaks.

Most outbreaks occurred in primary schools, which is consistent with a previous study on norovirus outbreaks in China in 2014–2017 (13). Kindergarten has a higher *TAR* and transmissibility than other types of schools for the following reasons: (1) Studies have found that students in kindergartens and primary schools are more likely to vomit when infected with norovirus (59), and some children in kindergartens may become infected with the virus by going around when vomiting occurs due to poor hygiene awareness. (2) Kindergartens are small and therefore have a high population density and higher exposure among younger children. We found that the mean *TAR* of norovirus outbreaks was highest in outbreaks with <1,000 susceptible people, and of the 61 outbreaks reported in Jiangsu Province, each with <1,000 susceptible people, the highest proportion of outbreaks occurred in kindergartens, so it can be assumed that small crowded places such as kindergartens are more likely to cause the spread of the outbreak. (3) Different types of schools are composed of students of different ages. Since children under 5 years of age are most susceptible (60), and most of the students in kindergartens in China are children aged 3–6 years old, norovirus outbreaks are more likely to occur in kindergartens.

Jiangsu Provincial Center for Disease Control and Prevention has undertaken a national foodborne and waterborne norovirus outbreak surveillance program. Once an outbreak of norovirus occurs, the corresponding outbreak investigation will be started, the cases, water and food will be sampled, and the prevention and control measures will be implemented. However, due to the high economic level and satisfactory sanitary conditions in Jiangsu Province, norovirus outbreaks caused by water or food are rare, accounting for only eight (3.9%) of all reported outbreaks. Among them, water source outbreaks are caused by barreled water contamination rather than pipe network contamination, and foodborne outbreaks are caused by a small number of kitchen workers infecting and polluting the food, and not from the food itself. These conditions led to a small significant difference in *TAR* among outbreaks of different transmission routes.

The genetic diversity of noroviruses can be reflected by differences in epidemiology (61). Surveillance of norovirus genotypes in our study showed that the major genotypes of noroviruses in Jiangsu province change every 2–3 years. The following characteristics of the changes in the genotypes causing norovirus outbreaks have been noted: since 2002, GII.4 has been the predominant genotype in norovirus outbreaks in many countries. In the winter of 2014, the predominant norovirus strain in China changed to GII.17. Subsequently, in the winter of 2016 (2016–17), GII.2 [P16] became the predominant genotype causing norovirus outbreaks (56). Norovirus outbreaks caused by the GII.2 [P16] genotype were also found in other regions and countries outside mainland China, such as Hong Kong, Taiwan, Germany, Japan, France, the United States, and Australia. This genotype has also become the major genotype causing norovirus outbreaks in Hong Kong, Taiwan, and Germany (54). The changes in genotypes we observed in Jiangsu province are temporally consistent with the changes in genotypes throughout China. Epidemiological surveillance and vaccines are essential to control human pathogens (62). However, it has been found that different genotypes show different evolutionary patterns, and the production of new variants of GII.4 genotype every 2–3 years will always lead to a population that is always susceptible to GII.4 genotype of norovirus. Once an immune barrier is established, individuals will no longer be susceptible to the GII.2 virus, which is classified as a static virus (63, 64). Based on this view, our future studies intend to observe this through continuous monitoring of genotypes and changes in population incidence levels in combination with other factors.

The mean R_{unc} of norovirus outbreaks in Jiangsu Province was 12.2, which was close to the data from a previous study on outbreaks (2 to >14) (65). In our previous study, we used the model fitting method to adjust the value of κ (interpersonal transmission coefficient) to be closer to 0. Finally, we obtained transmissibility of 1.94 for norovirus outbreaks in the community, 3.44 for transmissibility in schools and 4.91 for transmissibility by water (26); these results were all smaller compared to the values obtained in the current study. This difference can be explained by another study in which researchers found that the transmissibility of norovirus was largely influenced by the structure of the model and the weight of asymptomatic infections (66). We, therefore, considered that differences in parameter values in the model and differences in sample size could be responsible for the higher transmissibility of norovirus outbreaks in Jiangsu Province.

According to our research, further improvements are required in the sensitivity monitoring in northern Jiangsu. According to the epidemiological characteristics of norovirus in Jiangsu Province, the surveillance of norovirus outbreaks should be strengthened in all seasons of the year. Furthermore, health education and health promotion should be accomplished before autumn and winter where the incidence of norovirus is high, especially in rural areas. For schools, kindergartens

have the highest transmissibility, followed by primary schools. This phenomenon mainly considers the immunological differences between age groups and health habits. Older students who are not in kindergartens and primary schools, as well as teachers, should be more aware of norovirus and need to know how to handle vomit properly to prevent the spread of aerosols. Also, students who have gathered should be evacuated promptly and kept away from the vomit. Furthermore, suspected cases of norovirus infection should be isolated, especially kitchen staff. Finding out changes of dominant genotype has occurred is important for exploring the epidemiological characteristics of norovirus outbreaks in Jiangsu Province, however, not every positive specimen collected in an outbreak met the criteria for genetic sequencing and could be used to determine the genotype of the norovirus causing the outbreak. Therefore, tracking changes in the major genotypes causing outbreaks requires further surveillance.

CONCLUSION

The number of norovirus outbreaks in Jiangsu Province has generally been on the rise during the follow-up period, so prevention and control remain an important issue. Currently, factors such as year, month, season, region, urban and rural type, place type, the population of outbreak sites, route of transmission, and virus genotype significantly impact the *TAR* of norovirus outbreaks; notably, different types of schools influence transmissibility. The dominant genotype of the virus will change every 3 years and surveillance of norovirus genotypes should be further strengthened to explore the transmissibility of different genotypes. Our study explored how these factors affect the *TAR* and transmissibility of norovirus outbreaks in Jiangsu Province, suggesting current priorities for the prevention and control of norovirus outbreaks in Jiangsu Province and future issues to be addressed in the surveillance of outbreaks.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

CB, QL, TC, JA, YZ, JF, and XC contributed to conception and design of the study. JA, JF, XC, XZ, HJ, and WL collected data. TC, QL, YZ, JA, JR, TY, YW, JX, XL, MY, SL, and XG analyzed the data. CB, TC, JA, YZ, JF, and XC wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was partly supported by the Key Medical Discipline of Epidemiology (No. ZDXK A2016008), the National Major S&T Projects (No. 2018ZX10714-002), the Jiangsu Provincial Medical Youth Talent (No. QNRC2016542), the Project of Jiangsu Commission of health (Z2019006), the NHC Key Laboratory of Echinococcosis Prevention and Control (No. 2020WZK2001), and project S202110384382 supported by XMU Training Program of Innovation and Entrepreneurship for Undergraduates.

ACKNOWLEDGMENTS

The authors thank all study participants for providing the data and the field investigators for collecting the data.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.786096/full#supplementary-material

REFERENCES

1. Kapikian AZ, Wyatt RG, Dolin R, Thornhill TS, Kalica AR, Chanock RM. Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis. *J Virol.* (1972) 10:1075–81. doi: 10.1128/jvi.10.5.1075-1081.1972
2. Kapikian AZ. The discovery of the 27-nm Norwalk virus: an historic perspective. *J Infect Dis.* (2000) 181:S295–302. doi: 10.1086/315584
3. Chhabra P, de Graaf M, Parra GI, Chan MC, Green K, Martella V, et al. Updated classification of norovirus genogroups and genotypes. *J Gen Virol.* (2019) 100:1393–406. doi: 10.1099/jgv.0.001318
4. Mattison CP, Cardemil CV, Hall AJ. Progress on norovirus vaccine research: public health considerations and future directions. *Expert Rev Vaccines.* (2018) 17:773–84. doi: 10.1080/14760584.2018.1510327
5. Saupe AA, Rounds J, Sorenson A, Hedeen N, Bagstad E, Reinberg R, et al. Outbreak of norovirus gastroenteritis associated with ice cream contaminated by frozen raspberries from China; Minnesota, USA, 2016. *Clin Infect Dis.* (2021) 73:e3701–7. doi: 10.1093/cid/ciaa821
6. Zhou HL, Zhen SS, Wang JX, Zhang CJ, Qiu C, Wang SM, et al. Burden of acute gastroenteritis caused by norovirus in China: A systematic review. *J Infect.* (2017) 75:216–24. doi: 10.1016/j.jinf.2017.06.004
7. de Rougemont A, Ambert-Balay K, Belliot G, Pothier P. [Norovirus infections: an overview]. *Med Sci.* (2010) 26:73–8. doi: 10.1051/medsci/201026173
8. Barclay L, Park GW, Vega E, Hall A, Parashar U, Vinjé J, et al. Infection control for norovirus. *Clin Microbiol Infect.* (2014) 20:731–40. doi: 10.1111/1469-0691.12674
9. Lindsay L, Wolter J, De Coster I, Van Damme P, Verstraeten T, A. decade of norovirus disease risk among older adults in upper-middle and high income countries: a systematic review. *BMC Infect Dis.* (2015) 15:425. doi: 10.1186/s12879-015-1168-5
10. Li B, Xiao D, Li Y, Wu X, Qi L, W. Tang, et al. Epidemiological analysis of norovirus infectious diarrhea outbreaks in Chongqing, China, from 2011 to 2016. *J Infect Public Health.* (2020) 13:46–50. doi: 10.1016/j.jiph.2019.06.019
11. Gao Z, Liu B, Yan H, Li W, Jia L, Tian Y, et al. Norovirus outbreaks in Beijing, China, from 2014 to 2017. *J Infect.* (2019) 79:159–66. doi: 10.1016/j.jinf.2019.05.019
12. Li J, Gao X, Ye YL, Wan T, Zang H, Mo PH, et al. An acute gastroenteritis outbreak associated with person-to-person transmission in a primary school

in Shanghai: first report of a GI5 norovirus outbreak in China. *BMC Infect Dis.* (2018) 18:316. doi: 10.1186/s12879-018-3224-4
13. Lian Y, Wu S, Luo L, Lv B, Liao Q, Li Z, et al. Epidemiology of norovirus outbreaks reported to the public health emergency event surveillance system, China, 2014–2017. *Viruses*. (2019) 11:342. doi: 10.3390/v11040342
14. Allen DJ, Harris JP. Methods for ascertaining norovirus disease burdens. *Hum Vaccin Immunother.* (2017) 13:2630–6. doi: 10.1080/21645515.2017.1360455
15. Santos VS, Gurgel RQ, Cavalcante SM, Kirby A, Café LP, Souto MJ, et al. Acute norovirus gastroenteritis in children in a highly rotavirus-vaccinated population in Northeast Brazil. *J Clin Virol.* (2017) 88:33–8. doi: 10.1016/j.jcv.2016.10.015
16. Mans J, Armah GE, Steele AD, Taylor MB. Norovirus Epidemiology in Africa: a review. *PLoS ONE.* (2016) 11:e0146280. doi: 10.1371/journal.pone.0146280. [Epub ahead of print].
17. Pringle K, Lopman B, Vega E, Vinje J, Parashar UD, Hall AJ. Noroviruses: epidemiology, immunity and prospects for prevention. *Future Microbiol.* (2015) 10:53–67. doi: 10.2217/fmb.14.102
18. Fang X, Ai J, Liu W, Ji H, Zhang X, Peng Z, et al. Epidemiology of infectious diarrhoea and the relationship with etiological and meteorological factors in Jiangsu Province, China. *Sci Rep.* (2019) 9:19571. doi: 10.1038/s41598-019-56207-2
19. Fu JG, Shi C, Xu C, Lin Q, Zhang J, Yi QH, et al. Outbreaks of acute gastroenteritis associated with a re-emerging GII.P16-GII.2 norovirus in the spring of 2017 in Jiangsu, China. *PLoS ONE.* (2017) 12:e0186090. doi: 10.1371/journal.pone.0186090
20. Shi C, Feng WH, Shi P, Ai J, Guan HX, Sha D, et al. An acute gastroenteritis outbreak caused by GII17 norovirus in Jiangsu Province, China. *Int J Infect Dis.* (2016) 49:30–2. doi: 10.1016/j.ijid.2016.05.004
21. Fu J, Bao C, Huo X, Hu J, Shi C, Lin Q, et al. Increasing recombinant strains emerged in norovirus outbreaks in Jiangsu, China: 2015-2018. *Sci Rep.* (2019) 9:20012. doi: 10.1038/s41598-019-56544-2
22. Fu JG, Shi C, Sha D, Shi P, Bao CJ Ai J. [Genetic characterization of norovirus isolated in an outbreak of gastroenteritis in Jiangsu province]. *Zhonghua Liu Xing Bing Xue Za Zhi.* (2018) 39:72–4. doi: 10.3760/cma.j.issn.0254-6450.2018.01.015
23. Petrignani M, van Beek J, Borsboom G, Richardus JH, Koopmans M. Norovirus introduction routes into nursing homes and risk factors for spread: a systematic review and meta-analysis of observational studies. *J Hosp Infect.* (2015) 89:163–78. doi: 10.1016/j.jhin.2014.11.015
24. Geng Q, Shen Y, Shi P, Gao YM, Feng WH, Xiao Y, et al. Epidemiologic features and influencing factors of norovirus outbreaks in the City of Wuxi, China from 2014 to 2018. *Am J Trop Med Hyg.* (2021). doi: 10.4269/ajtmh.20-1371
25. Wu CY, Chi H, Liu CC, Huang YC, Huang YC, Lin HC, et al. Clinical characteristics and risk factors for children with norovirus gastroenteritis in Taiwan. *J Microbiol Immunol Infect.* (2021) 54:909–17. doi: 10.1016/j.jmii.2020.07.013
26. Chen T, Gu H, Leung RK, Liu R, Chen Q, Wu Y, et al. Evidence-based interventions of norovirus outbreaks in China. *BMC Public Health.* (2016) 16:1072. doi: 10.1186/s12889-016-3716-3
27. Liao Q, Ran L, Jin M, Cui S, Yuan J, Ma H, et al. [Guidelines on outbreak investigation, prevention and control of norovirus infection (2015)]. *Zhonghua Yu Fang Yi Xue Za Zhi.* (2016) 50:7–16. doi: 10.3760/cma.j.issn.0253-9624.2016.01.003
28. Fu J,Ai J,Jin M, Jiang C, Zhang J, Shi C, et al. Emergence of a new GII.17 norovirus variant in patients with acute gastroenteritis in Jiangsu, China, September 2014 to March 2015. *Euro Surveill.* (2015) 20:21157. doi: 10.2807/1560-7917.ES2015.20.24.21157
29. Cannon JL, Barclay L, Collins NR, Wikswo ME, Castro CJ, Magaña LC, et al. Genetic and epidemiologic trends of norovirus outbreaks in the United States from 2013 to 2016 demonstrated emergence of novel GII.4 recombinant viruses. *J Clin Microbiol.* (2017) 55:2208–21. doi: 10.1128/JCM.00455-17
30. Kojima S, Kageyama T, Fukushi S, Hoshino FB, Shinohara M, Uchida K, et al. Genogroup-specific PCR primers for detection of Norwalk-like viruses. *J Virol Methods.* (2002) 100:107–14. doi: 10.1016/S0166-0934(01)00404-9
31. Kumar S, Stecher G. Tamura K. MEGA7: molecular evolutionary genetics analysis version 70 for bigger datasets. *Mol Biol Evol.* (2016) 33:1870–4. doi: 10.1093/molbev/msw054
32. Tsang TK, Chen TM, Longini IM Jr, Halloran ME, Wu Y, Yang Y. Transmissibility of norovirus in urban versus rural households in a large community outbreak in China. *Epidemiology.* (2018) 29:675–83. doi: 10.1097/EDE.0000000000000855
33. Caul EO. Small round structured viruses: airborne transmission and hospital control. *Lancet.* (1994) 343:1240–2. doi: 10.1016/S0140-6736(94)92146-6
34. Hutson AM, Atmar RL, Estes MK. Norovirus disease: changing epidemiology and host susceptibility factors. *Trends Microbiol.* (2004) 12:279–87. doi: 10.1016/j.tim.2004.04.005
35. Atmar RL, Opekun AR, Gilger MA, Estes MK, Crawford SE, Neill FH, et al. Norwalk virus shedding after experimental human infection. *Emerg Infect Dis.* (2008) 14:1553–7. doi: 10.3201/eid1410.080117
36. Atmar RL, Estes MK. The epidemiologic and clinical importance of norovirus infection. *Gastroenterol Clin North Am.* (2006) 35:275–90, viii. doi: 10.1016/j.gtc.2006.03.001
37. Kaplan JE, Schonberger LB, Varano G, Jackman N, Bied J, Gary GW. An outbreak of acute nonbacterial gastroenteritis in a nursing home. Demonstration of person-to-person transmission by temporal clustering of cases. *Am J Epidemiol.* (1982) 116:940–8. doi: 10.1093/oxfordjournals.aje.a113496
38. Rockx B, De Wit M, Vennema H, Vinjé J, De Bruin E, Van Duynhoven Y, et al. Natural history of human calicivirus infection: a prospective cohort study. *Clin Infect Dis.* (2002) 35:246–53. doi: 10.1086/341408
39. Lopman BA, Reacher MH, Vipond IB, Sarangi J, Brown DW. Clinical manifestation of norovirus gastroenteritis in health care settings. *Clin Infect Dis.* (2004) 39:318–24. doi: 10.1086/421948
40. Kaplan JE, Feldman R, Campbell DS, Lookabaugh C, Gary GW. The frequency of a Norwalk-like pattern of illness in outbreaks of acute gastroenteritis. *Am J Public Health.* (1982) 72:1329–32. doi: 10.2105/AJPH.72.12.1329
41. Graham DY, Jiang X, Tanaka T, Opekun AR, Madore HP, Estes MK. Norwalk virus infection of volunteers: new insights based on improved assays. *J Infect Dis.* (1994) 170:34–43. doi: 10.1093/infdis/170.1.34
42. Phillips G, Lopman B, Tam CC, Iturriza-Gomara M, Brown D, Gray J. Diagnosing norovirus-associated infectious intestinal disease using viral load. *BMC Infect Dis.* (2009) 9:63. doi: 10.1186/1471-2334-9-63
43. Chen T, Leung RK, Zhou Z, Liu R, Zhang X, Zhang L. Investigation of key interventions for shigellosis outbreak control in China. *PLoS ONE.* (2014) 9:e95006. doi: 10.1371/journal.pone.0095006
44. Mattison K, Karthikeyan K, Abebe M, Malik N, Sattar SA, Farber JM, et al. Survival of calicivirus in foods and on surfaces: experiments with feline calicivirus as a surrogate for norovirus. *J Food Prot.* (2007) 70:500–3. doi: 10.4315/0362-028X-70.2.500
45. DallingJ. A review of environmental contamination during outbreaks of Norwalk-like virus. *Br J Infect Control.* (2004) 5:9–13. doi: 10.1177/14690446040050020301
46. D'Souza DH, Sair A, Williams K, Papafragkou E, Jean J, Moore C, et al. Persistence of caliciviruses on environmental surfaces and their transfer to food. *Int J Food Microbiol.* (2006) 108:84–91. doi: 10.1016/j.ijfoodmicro.2005.10.024
47. Wu HM, Fornek M, Schwab KJ, Chapin AR, Gibson K, Schwab E, et al. A norovirus outbreak at a long-term-care facility: the role of environmental surface contamination. *Infect Control Hosp Epidemiol.* (2005) 26:802–10. doi: 10.1086/502497
48. Jones EL, Kramer A, Gaither M, Gerba CP. Role of fomite contamination during an outbreak of norovirus on houseboats. *Int J Environ Health Res.* (2007) 17:123–31. doi: 10.1080/09603120701219394
49. Gallimore CI, Taylor C, Gennery AR, Cant AJ, Galloway A, Iturriza-Gomara M, et al. Environmental monitoring for gastroenteric viruses in a pediatric primary immunodeficiency unit. *J Clin Microbiol.* (2006) 44:395–9. doi: 10.1128/JCM.44.2.395-399.2006
50. Kuusi M, Nuorti JP, Maunula L, Minh Tran NN, Ratia M, Karlsson J, et al. A prolonged outbreak of Norwalk-like calicivirus (NLV) gastroenteritis in a rehabilitation centre due to environmental contamination. *Epidemiol Infect.* (2002) 129:133–8. doi: 10.1017/S0950268802007276
51. Cheesbrough JS, Green J, Gallimore CI, Wright PA, Brown DW. Widespread environmental contamination with Norwalk-like viruses (NLV) detected in a prolonged hotel outbreak of gastroenteritis. *Epidemiol Infect.* (2000) 125:93–8. doi: 10.1017/S095026889900432X
52. Green J, Wright PA, Gallimore CI, Mitchell O, Morgan-Capner P, Brown DW. The role of environmental contamination with small

round structured viruses in a hospital outbreak investigated by reverse-transcriptase polymerase chain reaction assay. *J Hosp Infect.* (1998) 39:39–45. doi: 10.1016/S0195-6701(98)90241-9
53. Ao Y, Wang J, Ling H, He Y, Dong X, Wang X, et al. Norovirus GII.P16/GII.2-associated gastroenteritis, China, 2016. *Emerg Infect Dis.* (2017) 23:1172–5. doi: 10.3201/eid2307.170034
54. Ao Y, Cong X, Jin M, Sun X, Wei X, Wang J, et al. Genetic analysis of reemerging GII.P16-GII.2 noroviruses in 2016-2017 in China. *J Infect Dis.* (2018) 218:133–43. doi: 10.1093/infdis/jiy182
55. Harris JP. Norovirus surveillance: an epidemiological perspective. *J Infect Dis.* (2016) 213(Suppl. 1):S8–11. doi: 10.1093/infdis/jiv452
56. Jin M, Wu S, Kong X, Xie H, Fu J, He Y, et al. Norovirus outbreak surveillance, China, 2016-2018. *Emerg Infect Dis.* (2020) 26:437–45. doi: 10.3201/eid2603.191183
57. Greig JD, Lee MB. Enteric outbreaks in long-term care facilities and recommendations for prevention: a review. *Epidemiol Infect.* (2009) 137:145–55. doi: 10.1017/S0950268808000757
58. Nicolay N, Boulet L, Le Bourhis-Zaimi M, Badjadj-Kab L, Henry L, Erouart S, et al. The role of dependency in a norovirus outbreak in a nursing home. *Eur Geriatr Med.* (2018) 9:837–44. doi: 10.1007/s41999-018-0120-9
59. Wang Y, Hao L, Pan L, Xue C, Liu Q, Zhao X, et al. Age, primary symptoms, and genotype characteristics of norovirus outbreaks in Shanghai schools in 2017. *Sci Rep.* (2018) 8:15238. doi: 10.1038/s41598-08-33724-0
60. Lopman BA, Adak GK, Reacher MH, Brown DW. Two epidemiologic patterns of norovirus outbreaks: surveillance in England and wales, 1992-2000. *Emerg Infect Dis.* (2003) 9:71–7. doi: 10.3201/eid0901.020175
61. van Beek J, de Graaf M, Al-Hello H, Allen DJ, Ambert-Balay K, Botteldoorn N, et al. Molecular surveillance of norovirus, 2005-16: an epidemiological analysis of data collected from the NoroNet network. *Lancet Infect Dis.* (2018) 18:545–53. doi: 10.1016/S1473-3099(18)30059-8
62. Green KY. Norovirus surveillance comes of age: the impact of NoroNet. *Lancet Infect Dis.* (2018) 18:482–3. doi: 10.1016/S1473-3099(18)30062-8
63. Parra GI, Squires RB, Karangwa CK, Johnson JA, Lepore CJ, Sosnovtsev SV, et al. Static and evolving norovirus genotypes: implications for epidemiology and immunity. *PLoS Pathog.* (2017) 13:e1006136. doi: 10.1371/journal.ppat.1006136
64. Zhou HL, Chen LN, Wang SM, Tan M, Qiu C, Qiu TY, et al. Prevalence and evolution of noroviruses between 1966 and 2019, implications for vaccinedesign. *Pathogens*. (2021) 10:1012. doi: 10.3390/pathogens10081012
65. Heijne JC, Teunis P, Morroy G, Wijkmans C, Oostveen S, Duizer E, et al. Enhanced hygiene measures and norovirus transmission during an outbreak. *Emerg Infect Dis.* (2009) 15:24–30. doi: 10.3201/eid1501.080299
66. Gaythorpe KAM, Trotter CL, Lopman B, Steele M, Conlan AJK. Norovirus transmission dynamics: a modelling review. *Epidemiol Infect.* (2018) 146:147–58. doi: 10.1017/S0950268817002692

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Medicine

SYSTEMATIC REVIEW
published: 13 January 2022
doi: 10.3389/fmed.2021.775698

Global Status of Bufavirus, Cosavirus, and Saffold Virus in Gastroenteritis: A Systematic Review and Meta-Analysis

Mohammad Hossein Razizadeh[1], *Alireza Khatami*[1]* *and Mohammad Zarei*[2,3]

[1] Faculty of Medicine, Department of Virology, Iran University of Medical Sciences, Tehran, Iran, [2] Renal Division, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, United States, [3] John B. Little Center for Radiation Sciences, Harvard T.H. Chan School of Public Health, Boston, MA, United States

Background: Bufavirus (BuV), Human Cosavirus (HCoSV), and Saffold (SAFV) virus are three newly discovered viruses and have been suggested as possible causes of gastroenteritis (GE) in some studies. The aim of the present study was to estimate the overall prevalence of viruses and their association with GE.

Methods: A comprehensive systematic search was conducted in Scopus, Web of Science, PubMed, and Google scholar between 2007 and 2021 to find studies on the prevalence of BuV, HCoSV, and SAFV viruses.

Result: Meta-analysis of the 46 included studies showed the low prevalence of BuV (1.%, 95% CI 0.6–1.5%), HCoSV (0.8%, 95% CI 0.4–1.5%), and SAFV (1.9%, 95% CI 1.1–3.1%) worldwide. Also, no significant association between these viruses and GE was observed. BuV was isolated from patients with GE in Africa, while SAFV was more common in Europe. BuV1 and BuV2 have the same prevalence between the three identified genotypes of BuV. HCoSV-C was the most prevalent genotype of HCoSV, and SAFV2 was the commonest genotype of SAFV. All of these viruses were more prevalent in children older than 5 years of age.

Conclusion: This was the first meta-analysis on the prevalence and association of BuV, HCoSV, and SAFV with GE. While no significant association was found between infection with these viruses and GE, we suggest more studies, especially with case-control design and from different geographical regions in order to enhance our knowledge of these viruses.

Keywords: Bufavirus, Saffold virus, Cosavirus, gastroenteritis, meta-analysis

OPEN ACCESS

Edited by:
Ali Taghipour,
Tarbiat Modares University, Iran

Reviewed by:
Mpumelelo Casper Rikhotso,
University of Venda, South Africa
Dawei Cui,
Zhejiang University School of Medicine, China

***Correspondence:**
Alireza Khatami
akh.alirezakhatami@gmail.com

Specialty section:
This article was submitted to Infectious Diseases – Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *14 September 2021*
Accepted: *29 October 2021*
Published: *13 January 2022*

Citation:
Razizadeh MH, Khatami A and Zarei M (2022) Global Status of Bufavirus, Cosavirus, and Saffold Virus in Gastroenteritis: A Systematic Review and Meta-Analysis. Front. Med. 8:775698. doi: 10.3389/fmed.2021.775698

INTRODUCTION

Gastroenteritis (GE) is one of the most common illnesses in both children and adults worldwide. The high importance of GE is due to both high morbidity and mortality and also the financial burdens of the disease. Children, the elderly, and immunocompromised individuals are at higher risk of severe GE (1). Infectious agents, particularly viruses are the main cause of GE worldwide (2). Before the implication of Rotavirus vaccination, Rotavirus was the leading cause of viral GE, while other enteric viruses, such as Noroviruses, Astroviruses, and Human adenoviruses, are now the

most prevalent viruses causing GE (3). Besides the aforementioned enteric viruses, the list of enteric viruses is continuously growing due to the discovery of emerging viruses (4, 5). Since still 40% of cases of GE are of unknown etiology (6), these newly discovered viruses may likely be involved in causing the GE (7).

The *Parvoviridae* family consists of small, non-enveloped, icosahedral-shaped viruses, which have a single-stranded DNA genome. Members of this family can infect both vertebrates and invertebrates (8). For about 3 decades, Parvovirus B19 was taught to be the only human pathogen in this family (9). In 2005, Human bocavirus 1 was isolated from the nasopharyngeal swab of children with respiratory symptoms. Since 2009, three other types of the virus, named Human bocavirus 2–4, have been isolated from a stool specimen of children with or without GE (10). In 2012, the metagenomic survey of stool samples of children with acute diarrhea in Burkina Faso resulted in the discovery of a new member of this family, which was named Bufavirus (BuV) (9). Human BuVs belong to the genus *Protoparvovirus*, and, so far, three genotypes of Human BuV have been identified (11).

The *Picornaviridae* family contains non-enveloped, icosahedral-shaped viruses with a positive-sense single-stranded RNA genome (12). Unlike the *Parvoviridae*, viruses in the *Picornaviridae* family are not able to infect invertebrates (13). This family contains a growing number of viruses, which cause a variety of diseases that can affect different organs of the body. In 2007, a new member of this family was isolated from a child with a fever of an unknown origin in the United States. This virus was later named Saffold virus (SAFV); this name was derived from the lead author of the research, Morris Saffold Jones. Phylogenetic analysis showed that this virus is closely related to *theilovirus* species in the *Cardiovirus* genus of this family (14). Since then, eight genotypes of SAFV have been identified (15). The other virus in this family is the Cosavirus (CoSV), which was discovered in 2008 in pediatric patients with acute flaccid paralysis and later found in patients with GE (7). These three novel viruses were isolated from patients with different clinical and epidemiologic patterns (4). They were isolated from patients with GE (6, 16) and neurological disorders (17–19). While GE is a threat to global health, the causative agents of many cases still remained unclear (4). Therefore, we conducted this systematic review and meta-analysis to (1) elucidate the possible role of these viruses in development of GE and (2) understand the current epidemiologic pattern of these viruses in different parts of the world.

METHODS

Search Strategy

This systematic and meta-analysis review was performed using the recommendations of the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) (20). We comprehensively searched from multiple electronic databases, including Web of Science, PubMed, Google scholar, and Scopus. English-language-related articles published from January 2007 to April 2021 were searched by two investigators independently (AK and MZ) using the following keywords: "Bufavirus" OR "BuV" OR "novel human picornavirus" OR "Saffold virus" OR "SAFV" OR "HCosV" OR "Human Cosavirus" AND "prevalence" OR "epidemiology" OR "molecular prevalence" AND "acute gastroenteritis" OR "diarrhea" OR "gastroenteritis" OR "gastrointestinal complications. In addition, the reference list of all relevant articles and narrative reviews were retrieved in full to search for additional eligible studies. All selected studies were imported to the EndNote software versionX8 (Thomson Reuters, California) for criteria analysis.

Inclusion and Exclusion Criteria

The inclusion criteria for the studies were as follows: (1) All observational studies (case-control, cohort, and cross-sectional studies); (2) Published: 2007 to 2021 for SAFV, between 2012 and 2021 for BuV, and between 2008 and 2021 for HCosV; and (3) Studies reporting the molecular techniques of Bufavirus, Saffoldvirus, and Cosavirus among patients with GE across the world. Papers were excluded from this review if (1) Samples were selected entirely from patients with Bufavirus, Saffold virus, and Cosavirus; (2) Research provides incomplete data; and (3) Review articles, congress abstracts, conference papers, meta-analysis, or systematic reviews, and articles in languages other than English.

Data Extraction

The data were extracted from 46 selected studies by two researchers separately and independently, including the first author's name, location, year of publication, continent, number of investigated patients, number of isolated viruses, target gene, molecular technique, and genotypes. If necessary, any issue related to the selection of studies was resolved by the first and corresponding authors.

Data Synthesis and Statistical Analysis

We used a random-effect model to estimate the overall prevalence of the BuV, SAFV, and HCosV, and results are shown in the forest plot with a 95% confidence interval. Furthermore, evaluation of the prevalence of the viruses was performed on continental, country, diagnostic method, and age as well as gender subgroups. Also, the prevalence of the viruses and their association with GE were estimated and reported by odds ratio (OR). The Egger's test and I^2 statistic/Cochran's Q statistic were used to determining publication bias and heterogeneity assessments, respectively, and $p < 0.05$ was considered statistically significant. All analyses of the present study were performed with comprehensive meta-analysis (V2.2, Bio stat) software.

RESULTS

Search Results and Studies Characteristics

Following the initial search strategy in the aforementioned databases, 3,604 original related articles were identified (PubMed: 755, Scopus: 178, Web of Science: 156, Google scholar: 2,515). A total of 46, observational articles, which included 30 cross-sectional (BuV: 6, SAFV: 12, and HCosV: 12), seven case-control (BuV: 1, SAFV: 3, and HCosV: 3), and nine cohort (BuV: 5,

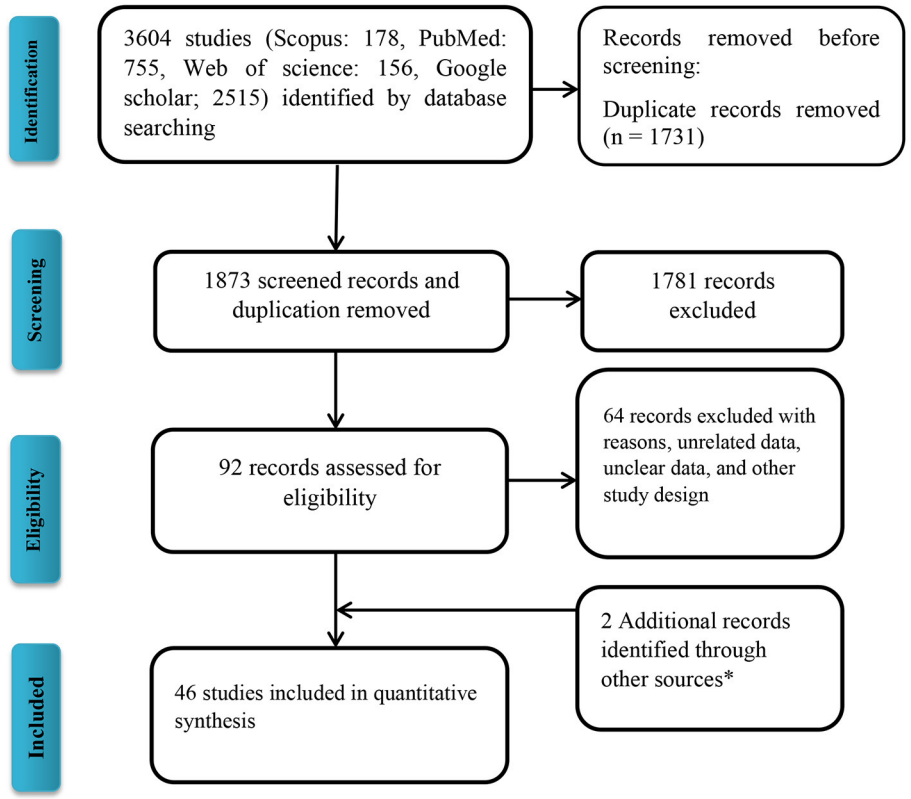

FIGURE 1 | Flow diagram of the literature search for studies included in the meta-analysis. *Including manual search and library records.

SAFV: 3, and HCosV: 1) studies were included based on our inclusion criteria. A summary of the research selection process and the reasons for exclusion is shown in **Figure 1**. In the case of Bufavirus, five articles were conducted in Europe, four in Asia, and three in Africa. About the Cosavirus, nine in Asia, four in Europe, one in Africa, and two articles were done in America. In the case of Saffold virus, 15 and three were performed in Asia Europe, respectively. Characteristics of the included 46 articles are shown in **Tables 1**–**3**.

Pooled Prevalence of Bufavirus in the Patients With Gastroenteritis

The total number of patients with GE included in this meta-analysis was 7,922 from children and adults based on 11 articles. The pooled prevalence of Bufavirus infection among patients with GE was 1.% (95% CI, 0.6–1.5%) based on a random-effects meta-analysis (**Figure 2**). In subgroup analysis by continent, the highest prevalence of Bufavirus was seen in Africa (1.4%, 95% CI, 0.5–4.1%) while the lowest prevalence was observed in Asia (0.7%, 95% CI, 0.2–2.1%) (**Table 4**). Highest prevalence of virus belongs to older than 5 years old subgroups (3.7%, 95% CI: 1.4–9.5%). As well, in three genotypes of BuV, BuV1 (1.%, 95% CI: 0.3–3.4%), and BuV2 (1.%, 95% CI: 0.1–6.9%) were of the same prevalence, while BuV3 (0.7%, 95% CI: 0.3–1.7%) was less prevalent.

The Association of Bufavirus With Gastroenteritis

In three data sets, the meta-analysis showed that Bufavirus was not associated with GE [OR: 2.191 (95% CI; 0.384–12.487), I^2: 0%] (**Figure 3**).

Pooled Prevalence of Saffold Virus in the Patients With Gastroenteritis

The results of analysis of Saffold virus based on random-effects meta-analysis are summarized in **Table 4**. Using random-effects meta-analysis, the pooled prevalence of Saffold virus in the studied patients was 1.9% (95% CI, 1.1–3.1%) (**Figure 4**). Among included studies, the maximum and minimum pooled prevalence of Saffold virus among patients with GE was found in Europe and Asia, respectively (2.9, 95% CI: 1.2–6.5% vs. 1.7, 95% CI: 0.9–3.1%) (**Table 5**). The highest prevalence of the virus was detected in children younger than 5 years of old (2.4%, 95% CI: 0.6–0.9). Among the eight genotypes of SAFV, SAFV-2 was the most prevalent genotype (1.%, 95% CI: 0.5–1.9%), and SAFV-4 was the least prevalent (0.2%, 95% CI: 0–1.2%) in patients with GE.

The Association of Saffold Virus With Gastroenteritis

Based on the meta-analysis of three case-control studies, there was no significant association between the Saffold virus and GE [OR: 0.768 (95% CI: 0.437–1.349), I^2: 0%] (**Figure 5**).

TABLE 1 | The general characterization of Bufavirus studies.

References	Study type	Country	Continent	Publishing year	Cases	Positive	Target	Method	Not distinguished Genotype	BuV1	BuV2	BuV3
Phan et al. (21)	Cross-sectional	Burkina Faso	Africa	2012	98	4	NS1	Nested RT-PCR		3	1	
Phan et al. (21)	Cross-sectional	Tunisia	Africa	2012	100	0	NS1	Nested RT-PCR				
Smits et al. (22)	Cross-sectional	Netherlands	Europe	2014	27	1	NS1	Real-time RT-PCR		1		
Vaisanen et al. (9)	Cross-sectional	Finland	Europe	2014	629	7	VP2	Real-time RT-PCR	7			
Yahiro et al. (23)	Cross-sectional	Bhutan	Asia	2014	393	3	NS1	Nested RT-PCR				3
Huang et al. (16)	Cross-sectional	China	Asia	2015	1877	9	NS1	Real-time RT-PCR		4		5
Altay et al. (24)	Case-control	Turkey	Europe	2015	583	8		RT-PCR				8
Chieochansin et al. (25)	Cohort	Thailand	Asia	2015	1414	1	NS1	Nested RT-PCR		1		
Chieochansin et al. (25)	Cohort	Thailand	Asia	2015	81	3	NS1	Nested RT-PCR		3		
Ayouni et al. (7)	Cohort	Tunisia	Africa	2016	203	2	NS1	Nested RT-PCR		2		
Vaisanen et al. (11)	Cohort	Finland	Europe	2016	410	3	NS1	Real-time RT-PCR	3			
Mohammad et al. (26)	Cross-sectional	Kuwait	Asia	2020	84	1		Multiplex RT-PCR				1
Dapra et al. (5)	Cohort	Italy	Europe	2021	160	0		Real-time RT-PCR				
Mohanraj et al. (27)	Cohort	Finland	Europe	2021	243	4	NS1	Multiplex real-time qPCR	4			
Mohanraj et al. (27)	Cohort	Finland	Europe	2021	386	3	NS1	Multiplex real-time qPCR	3			
Mohanraj et al. (27)	Cohort	Finland	Europe	2021	955	3	NS1	Multiplex real-time qPCR	3			
Mohanraj et al. (27)	Cohort	Latvia	Europe	2021	115	0	NS1	Multiplex real-time qPCR	0			
Mohanraj et al. (27)	Cohort	Malawi	Africa	2021	164	1	NS1	Multiplex real-time qPCR	1			

TABLE 2 | The general characterization of Saffold virus studies.

References	Study	Country	Continent	Publishing year	Cases	Positive	Target	Method	SAFV-1	SAFV-2	SAFV-3	SAFV-4	SAFV-6
Ren et al. (28)	Cross-sectional	China	Asia	2009	373	12	5′ UTR	Nested RT-PCR	12				
Khamrin et al. (29)	Cross-sectional	Thailand	Asia	2011	150	4	5′ UTR	Nested RT-PCR		4			
Dai et al. (30)	Case-control	China	Asia	2011	577	6	5′ UTR	Nested RT-PCR			3		
Zhang et al. (31)	Cohort	China	Asia	2012	2,013	12	5′ UTR	Real-time RT-PCR		4	5		
Khamrin et al. (32)	Cross-sectional	Japan	Asia	2013	454	7	5′ UTR	Nested RT-PCR		5	2		
Nielsen et al. (33)	Cohort	Denmark	Europe	2013	386	10	VP1	Real-time RT-PCR		10			
Yodmeeklin et al. (34)	Cross-sectional	Thailand	Asia	2015	608	9	5′ UTR	Nested RT-PCR	1	5	2	1	
Thongprachum et al. (35)	Cross-sectional	Japan	Asia	2017	751	4	5′ UTR	Multiplex RT-PCR					
Kumthip et al. (36)	Cross-sectional	Thailand	Asia	2017	73	1	5′ UTR	Nested RT-PCR					
Menage et al. (6)	Cross-sectional	Thailand	Asia	2017	1,093	18	5′ UTR	Nested RT-PCR	3	9			6
Li et al. (37)	Case-control	China	Asia	2017	461	7	VP1	Nested RT-PCR	3	4			
Dapra et al. (38)	Cross-sectional	Italy	Europe	2018	164	1		NR*					
Malasao et al. (39)	Cross-sectional	Thailand	Asia	2019	2,002	30		NR					
Kim et al. (40)	Cross-sectional	South Korea	Asia	2020	801	0		Multiplex RT-PCR					
Mohammad et al. (26)	Cross-sectional	Kuwait	Asia	2020	84	1		Metagenomics sequencing					
Vandesande et al. (41)	Cohort	Sweden	Europe	2021	209	11	5′ UTR	Semi-nested RT-PCR			1		
Yaghobi et al. (42)	Cross-sectional	Iran	Asia	2020	160	26	5′ UTR	RT-PCR					
Taghinejad et al. (43)	Cross-sectional	Iran	Asia	2020	160	11		RT-PCR					

**NR, Not reported.*

TABLE 3 | The general characterization of Cosavirus studies.

References	Study	Publishing year	Country	Continent	Cases	Positive
Nielsen et al. (33)	Cohort	2013	Denmark	Europe	386	0
Stocker et al. (44)	Case-control	2012	Brazil	America	359	13
Vizzi et al. (45)	Case-control	2021	Venezuela	America	82	5
Yu et al. (46)	Case-control	2017	China	Asia	461	8
Ayouni et al. (7)	Cross-sectional	2016	Tunisia	Africa	203	2
Dapra et al. (38)	Cross-sectional	2018	Italy	Europe	164	0
Dapra et al. (5)	Cross-sectional	2021	Italy	Europe	160	0
Khamrin et al. (47)	Cross-sectional	2012	Thailand	Asia	300	1
Khamrin et al. (48)	Cross-sectional	2014	Thailand	Asia	411	1
Kim et al. (40)	Cross-sectional	2020	South Korea	Asia	801	0
Menage et al. (6)	Cross-sectional	2017	Thailand	Asia	1,093	16
Mohammad et al. (26)	Cross-sectional	2020	Kuwait	Asia	84	1
Okitsu et al. (49)	Cross-sectional	2014	Japan	Asia	630	1
Rovida et al. (50)	Cross-sectional	2013	Italy	Europe	689	1
Thongprachum et al. (35)	Cross-sectional	2017	Japan	Asia	751	1
Kochjan et al. (51)	Cross-sectional	2016	Thailand	Asia	21	1

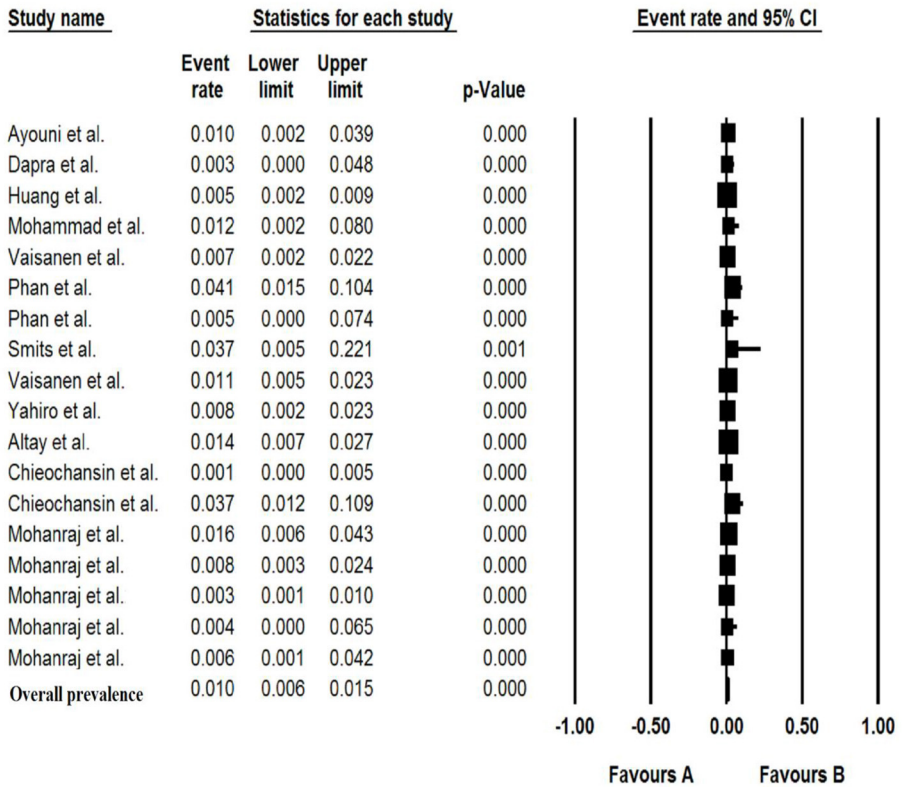

FIGURE 2 | Forest plot of the pooled prevalence for BuV.

Pooled Prevalence of Human Cosavirus in the Patients With Gastroenteritis

The total number of patients with GE included in this meta-analysis was 6,595 based on 16 included articles. Based on a random-effect meta-analysis, the pooled prevalence of the human Cosavirus infection among patients with GEs was 0.8% (95% CI, 0.4–1.5%) (**Figure 6**). In subgroup analysis by continent, the highest prevalence of Cosavirus was

TABLE 4 | The Bufavirus prevalence based on subgroups and studies heterogeneity.

Characteristics	Categories	Data sets	Pooled prevalence (%) (95% CI)	Heterogeneity		
				Q value	*P*-value	I^2%
Overall	–	18	1.0 (0.6–1.5)	35.005	0.006	51.435
Continent	Africa	4	1.4 (0.5–4.1)	5.486	0.139	45.319
	Asia	5	0.7 (0.2–2.1)	15.201	0.004	73.685
	Europe	9	1.0 (0.7–1.4)	9.203	0.325	13.071
Method	Nested RT-PCR	5	1.1 (0.4–3.1)	18.311	0.003	72.694
	Real-time RT-PCR	5	0.8 (0.4–1.4)	5.853	0.210	31.660
	multiplex real-time qPCR	5	0.7 (0.4–1.4)	4.975	0.290	19.599
Genotype	BuV1	6	1.0 (0.3–3.4)	27.351	0.000	81.719
	BuV2	1	1.0 (0.1–6.9)	0.000	1.000	0.000
	BuV3	4	0.7 (0.3–1.7)	8.548	0.036	0.501
Co–infection	NoV	6	0.3 (0.1–0.5)	4.103	0.535	0.000
	HBoV	2	0.3 (0.1–0.9)	0.078	0.780	0.000
	RoV	2	0.6 (0.2–2.2)	1.307	0.253	23.480
	AdV	1	1.0 (0.2–3.9)	0.000	1000	0.000
Age	Under 5	5	1.4 (0.6–2.9)	7.381	0.117	45.804
	Over 5	2	3.7 (1.4–9.5)	0.000	1.000	0.000
Sex	Male	4	0.9 (0.2–4.4)	12.447	0.006	75.898
	Female	4	0.6 (0.2–1.8)	4.279	0.233	29.883

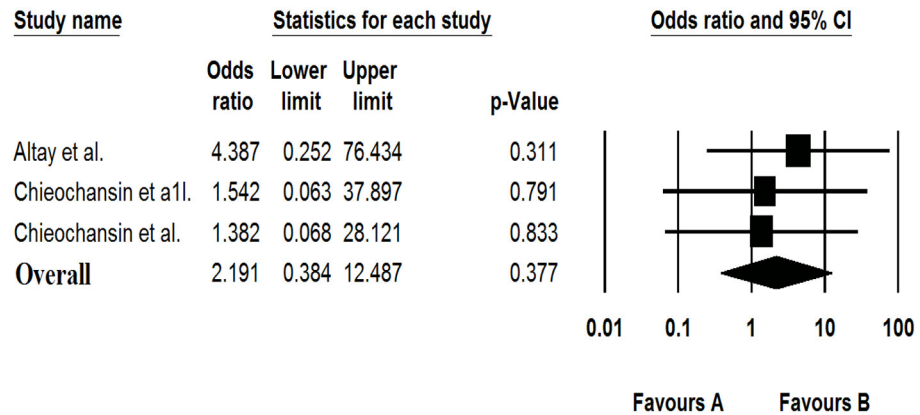

FIGURE 3 | Forest plot of odds ratios for the BuV based on case-control studies.

seen in America (4.2%, 95% CI, 2.6–6.6%), whereas Europe (0.2%, 95% CI, 0.1–0.7%) observed the lowest prevalence (**Table 6**).

The Association of Human Cosavirus With Gastroenteritis

Of the four included case-control studies, one study could not be analyzed due to zero values for cases and controls (33), and, according to the three analyzed studies, human Cosavirus was not associated with GE [OR: 0.730 (95% CI; 0.054–9.886), I^2: 0%] (**Figures 3**, **7**).

Publication Bias and Heterogeneity Assessment

The publication bias results were not significant for two viruses (SAFV and BuV) and significant for Cosavirus prevalence reports by applying Egger's regression test ($P = 0.1912$ for SAFV, $P = 0.5667$ for BuV, vs. $P = 0.0031$ for Cosavirus) (as shown in **Figure 8**). Also, the heterogeneity results of the studies according to the I^2 statistics and Cochran's Q statistics were statistically significant for BuV ($Q = 35.005$, $p < 0.006$, $I^2 = 51.435\%$), SAFV ($Q = 174.465$, $p < 0$, $I^2 = 90.256\%$), and Cosavirus ($Q = 28.29$, P = 0, $I^2 = 92.932$) (**Tables 4**–**6**).

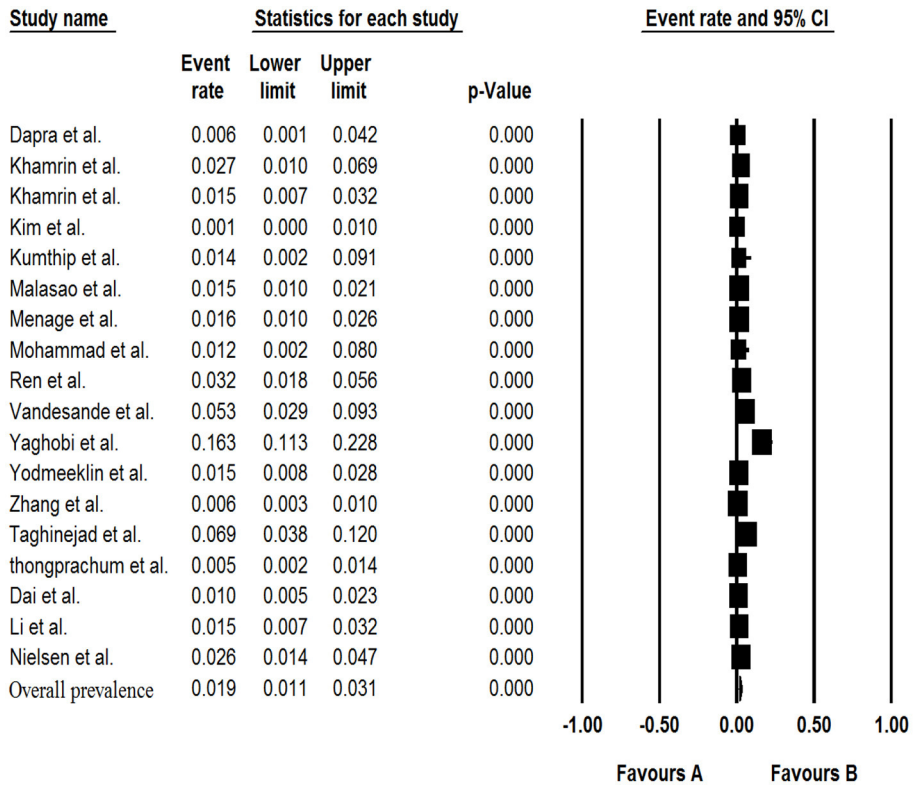

FIGURE 4 | Forest plot of the pooled prevalence for SAFV.

DISCUSSION

Rapid progressions in sequencing technologies, bioinformatics, and metagenomic have led to the discovery of new viruses in recent years. However, while some studies stated the isolation of new viruses from fecal samples of patients with GE, there is still no solid evidence of the association of these viruses with GE (4, 52, 53). They are often neglected in epidemiological studies as they cause milder or asymptomatic infection, and researchers have a higher tendency to detect common enteric viruses and other infectious agents in patients with GE (54–56). In the present meta-analysis, we investigated the role of three emerging discovered viruses in the development of GE. Our results show no association between infection with Bufavirus (OR; 2.91, 95% CI: 0.384–12.487), Cosavirus (OR; 0.73, 95% CI: 0.054–9.886), and Saffold virus (OR; 0.77, 95% CI: 0.44–1.35) with GE. Also, a low prevalence of BuV (1.%, 95% CI: 0.6–1.5%), HCoSV (0.8%, 95% CI: 0.4–1.5%), and SAFV (1.9%, 95% CI: 1.1–3.1%) was observed. In general, the prevalence of SAFV was higher than BuV, and the least prevalence was observed in the case of HCoSV. The highest prevalence of BuV was in Africa (1.4%, 95% CI: 0.5–4.1%), where it was discovered (21), and the least prevalence was in Asia (0.7%, 95% CI: 0.2–2.1%). This might be due to poor hygiene and lack of access to safe water in African countries. Given the fact that these viruses were detected in environmental and sewage samples from various parts of the world (57–62), they possibly transmit through the oral-fecal route.

About the three genotypes of BuV, BuV1, and BuV2 were of the same prevalence, while BuV3 was less common in patients with GE; this lower prevalence of BuV3 might be due to the later discovery of this genotype in 2014 (23). SAFV consists of eight genotypes, of which five (SAFV1-4 and 6) were found in the included studies. SAFV-2 was the most prevalent genotype, and SAFV-4 was the least prevalent in patients with GE. It should be pointed out that, although SAFV genotypes 5, 7, and 8 were not detected in the included studies, Blinkova et al. isolated them along with other genotypes in children with non-polio acute flaccid paralysis (63). Also, some of the included studies did not investigate the genotypes of isolated SAFVs. Therefore, we cannot conclude that they are not present in fecal samples of patients with GE. The genotype A of HCoSV was more frequently (0.5%, 95% CI: 0.1–2.1%) isolated from patients with GE. Other founded genotypes were Genotype D (0.2%, 95% CI: 0–0.7%) and C (0.1%, 95% CI: 0–0.6%).

The presence of common enteric viruses, such as Rotavirus (RoV), human bocavirus (HBoV), Adenovirus (AdV), and Norovirus (NoV), was observed in patients that are BuV and SAFV infected. According to the **Tables 4–6**, co-infection with NoV was more common in patients infected with SAFV than BuV. There was a similar situation in the case of HBoV in which more prevalence of this virus was seen in SAFV than patients

TABLE 5 | The Saffold virus prevalence based on subgroups and studies heterogeneity.

Characteristics	Categories	No. of Datasets	Pooled prevalence (%) (95% CI)	Heterogeneity		
				Q value	*P*-value	I^2%
Overall	–	18	1.9 (1.1–3.1)	174.465	0.000	90.256
Continent	Asia	15	1.7 (0.9–3.1)	165.693	0.000	91.553
	Europe	3	2.9 (1.2–6.5)	5.965	0.051	66.471
Genotype	SAFV-1	5	0.9 (0.3–2.6)	25.159	0.000	84.101
	SAFV-2	7	1.0 (0.5–1.9)	23.800	0.001	74.790
	SAFV-3	6	0.6 (0.2–1.5)	23.853	0.000	79.038
	SAFV-4	1	0.2 (0.0–1.2)	0.000	1.000	0.000
	SAFV-6	1	0.5 (0.2–1.2)	0.000	1.000	0.000
Co-infection	NoV	6	0.6 (0.3–1.0)	8.635	0.125	42.097
	HBoV	2	0.4 (0.1–1.5)	1.457	0.227	31.352
	RoV	8	0.4 (0.2–0.9)	19.395	0.007	63.909
	AdV	4	0.2 (0.1–0.5)	2.624	0.453	0.000
Method	Multiplex RT-PCR	2	0.3 (0.0–1.9)	2.052	0.152	51.263
	Nested RT-PCR	7	2.3 (1.5–3.5)	14.417	0.025	58.383
	RT-PCR	2	10.9 (4.6–24.)	6.505	0.011	84.627
Age	Under 5	8	1.6 (0.5–4.5)	70.138	0.000	90.020
	Over 5	3	2.4 (0.6–0.9)	4.183	0.124	52.184
Sex	Male	2	0.3 (0.0–2.2)	0.984	0.321	0.000
	Female	2	0.9 (0.0–19.7)	3.846	0.050	73.999

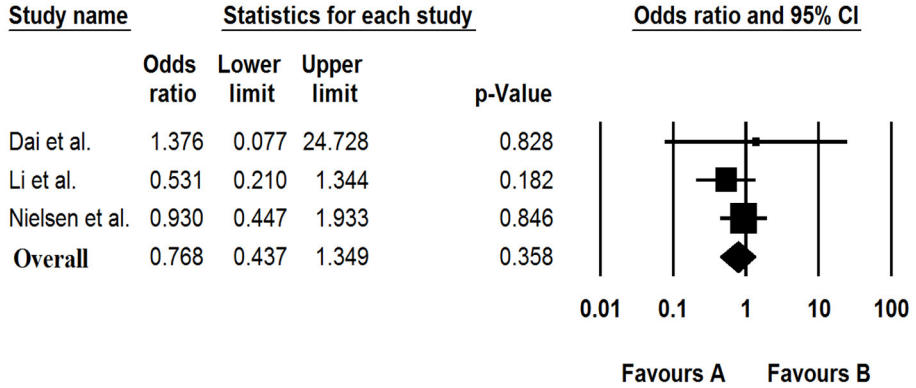

FIGURE 5 | Forest plot of odds ratios for the SAFV based on case-control studies.

who are BuV infected. Contrastingly, RoV infection was more frequent in patients infected with BuV than SAFV. Similarly, AdV infection was more common in patients with BuV than SAFV infection. EVs have the highest proportion of co-infection with HCoSV followed by AdVs, RoVs, and NoVs. The high rate of co-infection with classic enteric viruses may indicate the role of these viruses in causing symptoms in patients infected with these newly discovered viruses (6, 46). The other possible point that is against the pathologic role of these viruses in the development of GE is the low viral load in patients with GE, which might be due to transient infection and the lack of replication in the gastrointestinal tract (44). Also, the high presence of these viruses in healthy individuals raises the likelihood that they are a part of the human virome (6).

Three studied viruses can infect people of all age groups (16, 41). Our analysis showed that BuV and SAFV are more common in individuals older than 5 years of age. In contrast, HCoSV was more common in the children younger than 15 years old. While GE is known as a prevalent disease in children younger than 5 years of age and common enteric viruses such as RoV and NoV are mostly found in this age group (64, 65), interestingly, our analysis showed that these viruses are more prevalent in older patients. These results might be due to reason that outdoor activities further expose people to viral agents (52).

TABLE 6 | The Cosavirus prevalence based on subgroups and studies heterogeneity.

Characteristics	Categories	No. of Data sets	Pooled prevalence (%) (95% CI)	Heterogeneity		
				Q value	*P*-value	I^2%
Overall	–	16	0.8 (0.4–1.5)	28.29	0.000	92.932
WHO regions	Africa	1	1.0 (0.2–3.9)	0.000	1.000	0.000
	America	2	4.2 (2.6–6.6)	1.022	0.312	2.185
	Asia	9	0.7 (0.3–1.4)	21.240	0.007	62.335
	Europe	4	0.2 (0.1–0.7)	0.377	0.945	0.000
Genotype	HCoSV-A	3	0.5 (0.1–2.1)	6.292	0.043	68.213
	HCoSV-C	1	0.1 (0.0–0.6)	0.000	1.000	0.000
	HCoSV-D	2	0.2 (0.0–0.7)	0.837	0.360	0.000
Co-infection	NoV	2	0.2 (0.0–1.1)	1.420	0.233	29.561
	EV	3	0.7 (0.1–3.3)	5.932	0.052	66.286
	RoV	3	0.4 (0.2–0.8)	1.384	0.500	0.000
	AdV	5	0.6 (0.1–2.1)	9.329	0.053	57.122
Age	<5	10	0.5 (0.2–1.1)	21.031	0.013	57.207
	<15	7	1.2 (0.5–2.9)	18.564	0.005	67.680
	>15	2	0.4 (0.1–1.8)	0.319	0.517	0.000

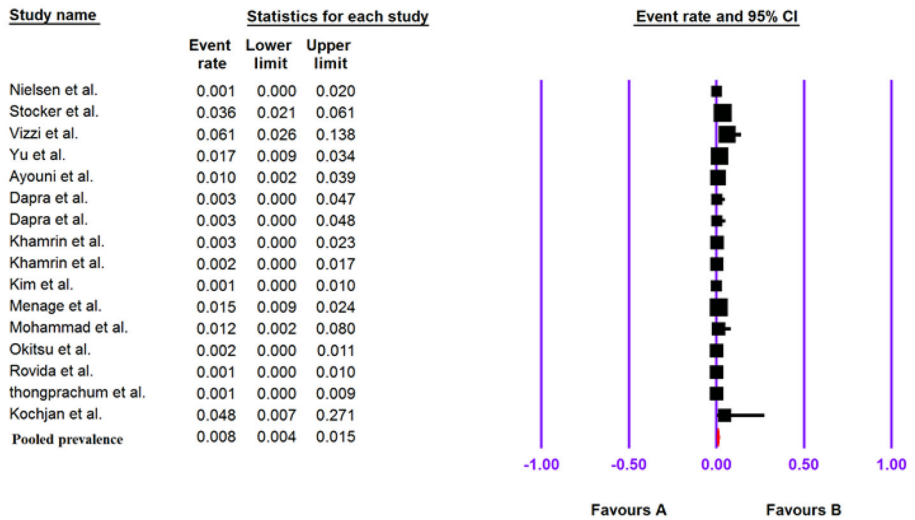

FIGURE 6 | Forest plot of the pooled prevalence for HCosV.

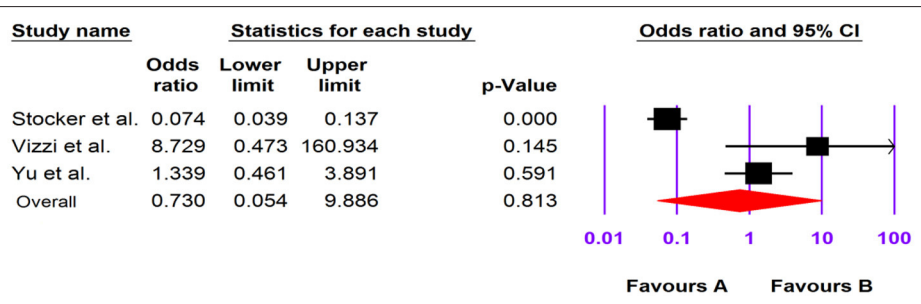

FIGURE 7 | Forest plot of odds ratios for the HCosV based on case-control studies.

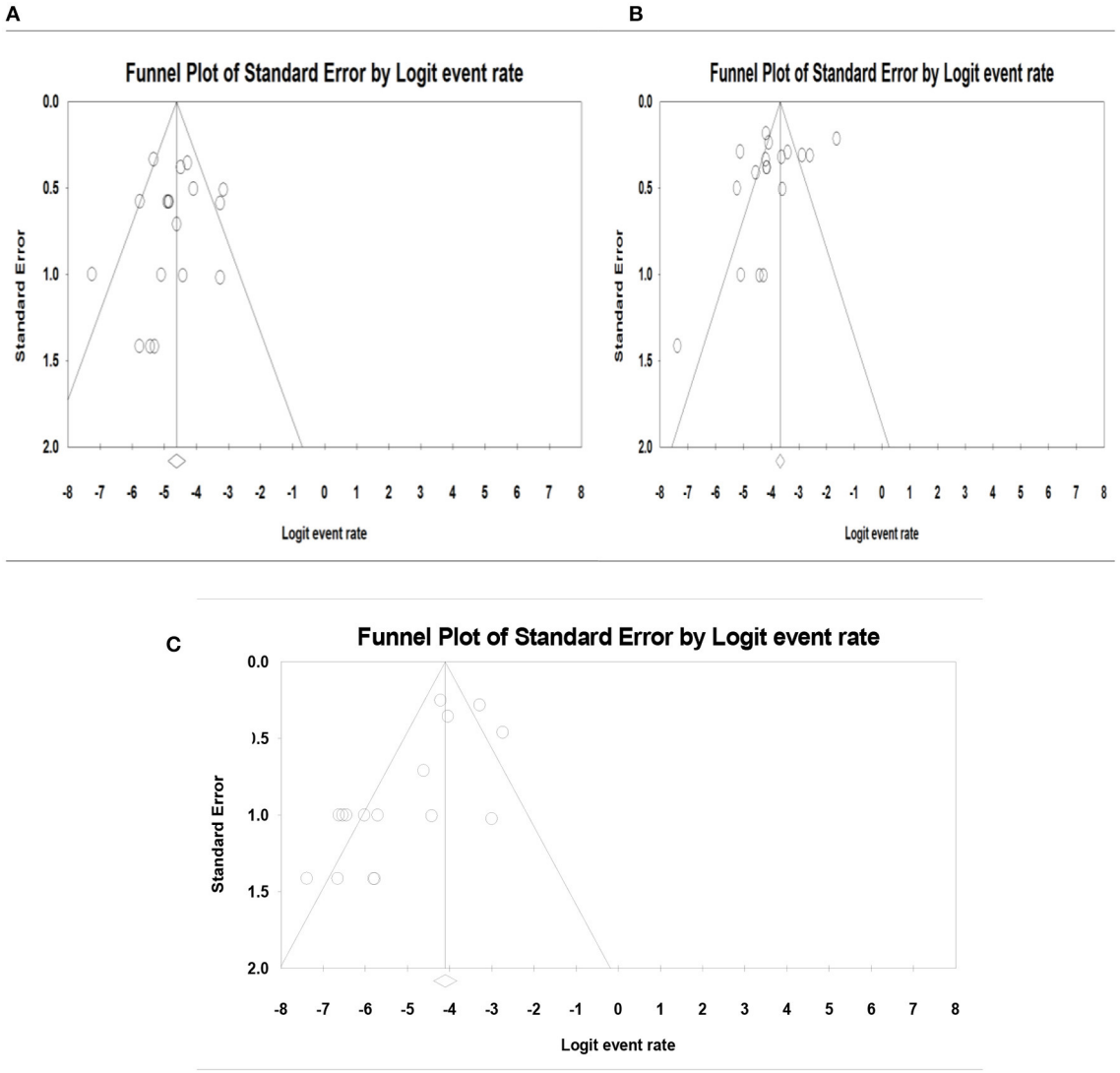

FIGURE 8 | Funnel plot for publication bias assessment in BuV **(A)**, SAFV **(B)**, and CosV **(C)**.

BuV and SAFV are differently distributed among males and females, while BuV is more prevalent in males than females; SAFV is more common in females (42). However, these slight differences do not implicate that these viruses have a higher tendency to infect people of a specific gender.

All included studies had a molecularly based diagnosis with relatively close sensitivity and specificity. However, in the case of SAFV, RT-PCR had the highest detection, while nested-PCR showed the highest detection rate for BuV. It is noteworthy to mention that it requires more studies on the sensitivity and specificity of these methods to conclude which one is more suitable.

The present study faced some limitations. There were a few studies on adults, and details of participants (gender, clinical signs, and age groups) were insufficient in some studies. The genotypes of the viruses were not reported from some studies, and also some of research conducted without a healthy control group. The prevalence of these viruses had not been reported in many countries and geographical areas. In addition, some of the included studies did not evaluate the co-infection of the novel viruses with common enteric viruses. In addition, the language limitations of many studies and lack of association assessments of genotypes and clinical signs were the other main limitations of the present study. Hence, we suggest further studies, especially in case-control design, and more comprehensive studies from different geographical areas to overcome these limitations.

CONCLUSION

Progression in the development of molecular and metagenomics methods has facilitated discovering and studying emerging

viruses. In the present meta-analysis, we investigated the prevalence and role of three recently discovered viruses in the development of GE. The pooled prevalence of three viruses was low, and neither was associated with GE. These results might be due to the few numbers of studies conducted. Therefore, we suggest more comprehensive studies with large cohorts of symptomatic and healthy patients in order to enhance our knowledge about these newly identified viruses. Also, we recommend *in vitro* studies to investigate the possible effects of these viruses on the gastrointestinal cell lines. In addition, the possible role of these emerging viruses in the etiology of other complications, such as respiratory symptoms, neurological diseases, and fever of an unknown origin, should not be neglected.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

MR and AK designed the study and collaborated in the manuscript writing. MR and MZ collaborated in the studies search, data extraction, and double checking. MZ helped in revision. All authors commented on the drafts of the manuscript and approved the final version of the article.

REFERENCES

1. Eckardt AJ, Baumgart DC. Viral gastroenteritis in adults. *Recent Patents Anti Infect Drug Discov*. (2011) 6:54–63. doi: 10.2174/157489111794407877
2. Kesheh MM, Khatami A, Saadati H, Jabbari M, Razizadeh MH, Fatemipour M, et al. *Salivirus Infection: Systematic Review and Meta-Analysis of Association With Gastrointestinal Symptoms in Children*. New York, NY: Wiley Online Library.
3. Arashkia A, Bahrami F, Farsi M, Nejati B, Jalilvand S, Nateghian A, et al. Molecular analysis of human adenoviruses in hospitalized children< 5 years old with acute gastroenteritis in Tehran, Iran. *J Med Virol*. (2019) 91:1930–6. doi: 10.1002/jmv.25539
4. Smits SL, Osterhaus AD, Koopmans MP. Newly identified viruses in human gastroenteritis: pathogens or not? *Pediatr Infect Dis J*. (2016) 35:104–7. doi: 10.1097/INF.0000000000000950
5. Daprà V, Galliano I, Montanari P, Zaniol E, Calvi C, Alliaudi C, et al. Bufavirus, Cosavirus, and Salivirus in diarrheal Italian infants. *Intervirology*. (2021) 64:165–8. doi: 10.1159/000514384
6. Menage L, Yodmeeklin A, Khamrin P, Kumthip K, Maneekarn N. Prevalence of human cosavirus and saffold virus with an emergence of saffold virus genotype 6 in patients hospitalized with acute gastroenteritis in Chiang Mai, Thailand, 2014–2016. *Infect Genet Evol*. (2017) 53:1–6. doi: 10.1016/j.meegid.2017.05.005
7. Ayouni S, Estienney M, Hammami S, Neji Guediche M, Pothier P, Aouni M, et al. Cosavirus, salivirus and bufavirus in diarrheal Tunisian infants. *PLoS ONE*. (2016) 11:e0162255. doi: 10.1371/journal.pone.0162255
8. Cotmore SF, Agbandje-McKenna M, Canuti M, Chiorini JA, Eis-Hubinger A-M, Hughes J, et al. ICTV virus taxonomy profile: parvoviridae. *J Gen Virol*. (2019) 100:367–8. doi: 10.1099/jgv.0.001212
9. Väisänen E, Kuisma I, Phan TG, Delwart E, Lappalainen M, Tarkka E, et al. Bufavirus in feces of patients with gastroenteritis, Finland. *Emerg Infect Dis*. (2014) 20:1077–9. doi: 10.3201/eid2006.131674
10. Christensen A, Kesti O, Elenius V, Eskola AL, Døllner H, Altunbulakli C, et al. Human bocaviruses and paediatric infections. *Lancet Child AdolescHealth*. (2019) 3:418–26. doi: 10.1016/S2352-4642(19)30057-4
11. Väisänen E, Paloniemi M, Kuisma I, Lithovius V, Kumar A, Franssila R, et al. Epidemiology of two human protoparvoviruses, bufavirus and tusavirus. *Sci Rep*. (2016) 6:39267. doi: 10.1038/srep39267
12. Zell R, Delwart E, Gorbalenya AE, Hovi T, King AMQ, Knowles NJ, et al. ICTV virus taxonomy profile: picornaviridae. *J Gen Virol*. (2017) 98:2421–2. doi: 10.1099/jgv.0.000911
13. Zell R. Picornaviridae—the ever-growing virus family. *Arch Virol*. (2018) 163:299–317. doi: 10.1007/s00705-017-3614-8
14. Tan SZK, Tan MZY, Prabakaran M. Saffold virus, an emerging human cardiovirus. *Rev Med Virol*. (2017) 27:e1908. doi: 10.1002/rmv.1908
15. Himeda T, Ohara Y. Saffold virus, a novel human cardiovirus with unknown pathogenicity. *J Virol*. (2012) 86:1292–6. doi: 10.1128/JVI.06087-11
16. Huang D-D, Wang W, Lu Q-B, Zhao J, Guo C-T, Wang H-Y, et al. Identification of bufavirus-1 and bufavirus-3 in feces of patients with acute diarrhea, China. *Sci Rep*. (2015) 5:1–4. doi: 10.1038/srep13272
17. Rezig D, Farhat EB, Touzi H, Meddeb Z, Salah AB, Triki H. Prevalence of human cosaviruses in Tunisia, North Africa. *J Med Virol*. (2015) 87:940–3. doi: 10.1002/jmv.24076
18. Altay Koçak A, Öcal M, Polat M, Kanik Yüksek S, Aktaş Tapisiz A, Tezer H, et al. [Multicenter investigation of bufavirus in the etiology of viral central nervous system infections of adults and children]. *Mikrobiyol Bul*. (2017) 51:191–4. doi: 10.5578/mb.54035
19. Naeem A, Hosomi T, Nishimura Y, Alam MM, Oka T, Zaidi SSZ, et al. Genetic diversity of circulating Saffold viruses in Pakistan and Afghanistan. *J Gen Virol*. (2014) 95:1945–57. doi: 10.1099/vir.0.066498-0
20. Moher D, Liberati A, Tetzlaff J, Altman DG, Group P. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PLoS Med*. (2009) 6:e1000097. doi: 10.1371/journal.pmed.1000097
21. Phan TG, Vo NP, Bonkoungou IJ, Kapoor A, Barro N, O'Ryan M, et al. Acute diarrhea in West African children: diverse enteric viruses and a novel parvovirus genus. *J Virol*. (2012) 86:11024–30. doi: 10.1128/JVI.01427-12
22. Smits SL, Schapendonk CM, van Beek J, Vennema H, Schürch AC, Schipper D, et al. New viruses in idiopathic human diarrhea cases, the Netherlands. *Emerg Infect Dis*. (2014) 20:1218. doi: 10.3201/eid2007.140190
23. Yahiro T, Wangchuk S, Tshering K, Bandhari P, Zangmo S, Dorji T, et al. Novel human bufavirus genotype 3 in children with severe diarrhea, Bhutan. *Emerg Infect Dis*. (2014) 20:1037. doi: 10.3201/eid2006.131430
24. Altay A, Yahiro T, Bozdayi G, Matsumoto T, Sahin F, Ozkan S, et al. Bufavirus genotype 3 in Turkish children with severe diarrhoea. *Clin Microbiol Infect*. (2015) 21:965.e1-4. doi: 10.1016/j.cmi.2015.06.006
25. Chieochansin T, Vutithanachot V, Theamboonlers A, Poovorawan Y. Bufavirus in fecal specimens of patients with and without diarrhea in Thailand. *Arch Virol*. (2015) 160:1781–4. doi: 10.1007/s00705-015-2441-z
26. Mohammad HA, Madi NM, Al-Nakib W. Analysis of viral diversity in stool samples from infants and children with acute gastroenteritis in Kuwait using Metagenomics approach. *Virol J*. (2020) 17:10. doi: 10.1186/s12985-020-1287-5
27. Mohanraj U, Jokinen M, Thapa RR, Paloniemi M, Vesikari T, Lappalainen M, et al. Human Protoparvovirus DNA and IgG in Children and Adults with and without Respiratory or Gastrointestinal Infections. *Viruses*. (2021) 13:483. doi: 10.3390/v13030483
28. Ren L, Gonzalez R, Xiao Y, Xu X, Chen L, Vernet G, et al. Saffold cardiovirus in children with acute gastroenteritis, Beijing, China. *Emerg Infect Dis*. (2009) 15:1509. doi: 10.3201/eid1509.081531
29. Khamrin P, Chaimongkol N, Nantachit N, Okitsu S, Ushijima H, Maneekarn N. Saffold cardioviruses in children with diarrhea, Thailand. *Emerg Infect Dis*. (2011) 17:1150. doi: 10.3201/eid1706.101983

30. Dai X, Yuan C, Yu Y, Zhao W, Yang Z. Molecular detection of Saffold virus in children in Shanghai, China. *J Clin Virol*. (2011) 50:186–7. doi: 10.1016/j.jcv.2010.11.004
31. Zhang X-A, Lu Q-B, Wo Y, Zhao J, Huang D-D, Guo C-T, et al. Prevalence and genetic characteristics of Saffold cardiovirus in China from 2009 to 2012. *Sci Rep*. (2015) 5:1–7. doi: 10.1038/srep07704
32. Khamrin P, Thongprachum A, Kikuta H, Yamamoto A, Nishimura S, Sugita K, et al. Three clusters of Saffold viruses circulating in children with diarrhea in Japan. *Infect Genet Evol*. (2013) 13:339–43. doi: 10.1016/j.meegid.2012.11.004
33. Nielsen ACY, Gyhrs ML, Nielsen LP, Pedersen C, Böttiger Gastroenteritis B, and the novel picornaviruses aichi virus, cosavirus, saffold virus, and salivirus in young children. *J Clin Virol*. (2013) 57:239–42. doi: 10.1016/j.jcv.2013.03.015
34. Yodmeeklin A, Khamrin P, Chuchaona W, Saikruang W, Malasao R, Chaimongkol N, et al. Saffold viruses in pediatric patients with diarrhea in Thailand. *J Med Virol*. (2015) 87:702–7. doi: 10.1002/jmv.24114
35. Thongprachum A, Khamrin P, Pham NTK, Takanashi S, Okitsu S, Shimizu H, et al. Multiplex RT-PCR for rapid detection of viruses commonly causing diarrhea in pediatric patients. *J Med Virol*. (2017) 89:818–24. doi: 10.1002/jmv.24711
36. Kumthip K, Khamrin P, Ushijima H, Maneekarn N. Multiple enterovirus genotypes circulating in children hospitalized with acute gastroenteritis in Thailand. *Infect Genet Evol*. (2017) 55:324–31. doi: 10.1016/j.meegid.2017.10.002
37. Li L-l, Liu N, Yu J-m, Ao Y-y, Li S, Stine OC, et al. Analysis of Aichi virus and Saffold virus association with pediatric acute gastroenteritis. *J Clin Virol*. (2017) 87:37–42. doi: 10.1016/j.jcv.2016.12.003
38. Daprà V, Montanari P, Rassu M, Calvi C, Galliano I, Bergallo M. Prevalence of human cosavirus and saffold virus in young children with gastroenteritis, Northern Italy. *Minerva Pediatr*. (2018). doi: 10.23736/s0026-4946.18.05219-2
39. Malasao R, Khamrin P, Kumthip K, Ushijima H, Maneekarn N. Molecular epidemiology and genetic diversity of human parechoviruses in children hospitalized with acute diarrhea in Thailand during 2011–2016. *Arch Virol*. (2019) 164:1743–52. doi: 10.1007/s00705-019-04249-2
40. Kim GR, Kim SH, Jeon GW, Shin JH. Prevalence of eleven infectious viruses causing diarrhea in Korea. *Jpn J Infect Dis*. (2020) 73:427–30. doi: 10.7883/yoken.JJID.2020.069
41. Vandesande H, Edman K, Rondahl E, Falkeborn T, Serrander L, Lindberg AM. Saffold virus infection in elderly people with acute gastroenteritis in Sweden. *J Med Virol*. (2021) 93:3980–4. doi: 10.1002/jmv.26452
42. Yaghobi TS, Bahrami H, Harzandi N, Asadi A, Shareghi M, Firouzjani MH, et al. First molecular detection of saffold virus in children with acute gastroenteritis in iran. *Novelty in Biomedicine*. 9:11–6.
43. Taghinejad M, Ghaderi M, Mousavi-Nasab SD. First molecular detection of aichivirus in pediatric patients with acute gastroenteritis in Iran. *Novelty Biomed*. (2020) 8:20–5. doi: 10.22037/nbm.v7i4.26729
44. Stöcker A, Souza BFdCD, Ribeiro TCM, Netto EM, Araujo LO, Corrêa JI, et al. Cosavirus infection in persons with and without gastroenteritis, Brazil. *Emerg Infect Dis*. (2012) 18:656. doi: 10.3201/eid1804.111415
45. Vizzi E, Fernández R, Angulo LA, Blanco R, Pérez C. Human cosavirus infection in HIV subjects with diarrhoea: persistent detection associated with fatal outcome. *J Clin Virol*. (2021) 139:104825. doi: 10.1016/j.jcv.2021.104825
46. Yu J-M, Ao Y-Y, Li L-L, Duan Z-J. Identification of a novel cosavirus species in faeces of children and its relationship with acute gastroenteritis in China. *Clin Microbiol Infect*. (2017) 23:550–4. doi: 10.1016/j.cmi.2017.02.018
47. Khamrin P, Chaimongkol N, Malasao R, Suantai B, Saikhruang W, Kongsricharoern T, et al. Detection and molecular characterization of cosavirus in adults with diarrhea, Thailand. *Virus Genes*. (2012) 44:244–6. doi: 10.1007/s11262-011-0700-y
48. Khamrin P, Maneekarn N. Detection and genetic characterization of cosavirus in a pediatric patient with diarrhea. *Arch Virol*. (2014) 159:2485–9. doi: 10.1007/s00705-014-2091-6
49. Okitsu S, Khamrin P, Thongprachum A, Nishimura S, Kalesaran AF, Takanashi S, et al. Detection and molecular characterization of human cosavirus in a pediatric patient with acute gastroenteritis, Japan. *Infect Genet Evol*. (2014) 28:125–9. doi: 10.1016/j.meegid.2014.09.019
50. Rovida F, Campanini G, Piralla A, Adzasehoun KMG, Sarasini A, Baldanti F. Molecular detection of gastrointestinal viral infections in hospitalized patients. Diagnostic microbiology and infectious disease. (2013) 77:231–5. doi: 10.1016/j.diagmicrobio.2013.07.020
51. Kochjan P, Khamrin P, Kumthip K, Maneekarn N. *Detection and Characterization of Human Bocavirus in Pediatric Patients with Acute Gastroenteritis in Chiang Mai, Thailand*.
52. Razizadeh MH, Khatami A, Zarei M. Global molecular prevalence and genotype distribution of Sapovirus in children with gastrointestinal complications: a systematic review and meta-analysis. *Rev Med Virol*. (2021) e2302. doi: 10.1002/rmv.2302
53. Kesheh MM, Khatami A, Saadati H, Jabbari M, Razizadeh MH, Fatemipour M, et al. Salivirus infection: systematic review and meta-analysis of association with gastrointestinal symptoms in children. *Rev Med Virol*. (2021) e2238. doi: 10.1002/rmv.2238
54. Taghipour A, Bahadory S, Badri M, Yadegar A, Mirsamadi ES, Mirjalali H, et al. A systematic review and meta-analysis on the co-infection of Helicobacter pylori with intestinal parasites: public health issue or neglected correlation? *Int J Environ Health Res*. (2020) 1–11. doi: 10.1080/09603123.2020.1798890
55. Taghipour A, Olfatifar M, Foroutan M, Bahadory S, Malih N, Norouzi M. Global prevalence of Cryptosporidium infection in rodents: a systematic review and meta-analysis. *Prev Vet Medi*. (2020) 105119. doi: 10.1016/j.prevetmed.2020.105119
56. Khatami A, Bahadory S, Ghorbani S, Saadati H, Zarei M, Soleimani A, et al. Two rivals or colleagues in the liver? Hepatit B virus and Schistosoma mansoni co-infections: a systematic review and meta-analysis. *Microbial Pathogen*. (2021) 104828. doi: 10.1016/j.micpath.2021.104828
57. López GR, Martinez LM, Freyre L, Freire MC, Vladimirsky S, Rabossi A, et al. Persistent detection of Cosavirus and Saffold cardiovirus in Riachuelo River, Argentina. *Food Environ Virol*. (2021) 13:64–73. doi: 10.1007/s12560-020-09451-z
58. Guerrero-Latorre L, Romero B, Bonifaz E, Timoneda N, Rusiñol M, Girones R, et al. Quito's virome: metagenomic analysis of viral diversity in urban streams of Ecuador's capital city. *Sci Total Environ*. (2018) 645:1334–43. doi: 10.1016/j.scitotenv.2018.07.213
59. Nantachit N, Khamrin P, Kumthip K, Malasao R, Maneekarn N. Molecular surveillance and genetic analyses of bufavirus in environmental water in Thailand. *Infect Genet Evol*. (2019) 75:104013. doi: 10.1016/j.meegid.2019.104013
60. Thongprachum A, Fujimoto T, Takanashi S, Saito H, Okitsu S, Shimizu H, et al. Detection of nineteen enteric viruses in raw sewage in Japan. *Infect Genet Evol*. (2018) 63:17–23. doi: 10.1016/j.meegid.2018.05.006
61. Bonanno Ferraro G, Mancini P, Veneri C, Iaconelli M, Suffredini E, Brandtner D, et al. Evidence of Saffold virus circulation in Italy provided through environmental surveillance. *Lett Appl Microbiol*. (2020) 70:102–8. doi: 10.1111/lam.13249
62. Aminipour M, Ghaderi M, Harzandi N. First occurrence of Saffold virus in sewage and river water samples in Karaj, Iran. *Food Environ Virol*. (2020) 12:75–80. doi: 10.1007/s12560-019-09415-y
63. Blinkova O, Kapoor A, Victoria J, Jones M, Wolfe N, Naeem A, et al. cardioviruses are genetically diverse and cause common enteric infections in South Asian children. *J Virol*. (2009) 83:4631–41. doi: 10.1128/JVI.02085-08
64. Bányai K, Estes MK, Martella V, Parashar UD. Viral gastroenteritis. *Lancet*. (2018) 392:175–86. doi: 10.1016/S0140-6736(18)31128-0
65. Nguyen GT, Phan K, Teng I, Pu J, Watanabe T. A systematic review and meta-analysis of the prevalence of norovirus in cases of gastroenteritis

in developing countries. *Medicine*. (2017) 96:e8139. doi: 10.1097/MD.0000000000008139
66. Ghaderi M. First molecular detection of Saffold virus in children with acute gastroenteritis in Iran. *Novelty Biomed*. (2021) 9:11–6. doi: 10.22037/nbm.vi.31662

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Medicine

REVIEW
published: 20 June 2022
doi: 10.3389/fmed.2022.890127

Gut Microbiota Shifting in Irritable Bowel Syndrome: The Mysterious Role of *Blastocystis* sp.

Alireza Olyaiee[1], *Amir Sadeghi*[2], *Abbas Yadegar*[3], *Elnaz Sadat Mirsamadi*[4]* and *Hamed Mirjalali*[3]*

[1] Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran, [2] Gastroenterology and Liver Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran, [3] Foodborne and Waterborne Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran, [4] Department of Microbiology, Faculty of Medicine, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran

Irritable bowel syndrome (IBS) is a chronic disorder, which its causative agent is not completely clear; however, the interaction between microorganisms and gastrointestinal (GI) epithelial cells plays a critical role in the development of IBS and presenting symptoms. During recent decades, many studies have highlighted the high prevalence of *Blastocystis* sp. in patients with IBS and suggested a probable role for this protist in this disease. Recent studies have documented changes in the gut microbiota composition in patients with IBS regarding the presence of *Blastocystis* sp., but it is not clear that either disturbance of the gut during GI disorders is a favorable condition for *Blastocystis* sp. colonization or the presence of this protist may lead to alteration in the gut microbiota in IBS patients. In this review, we comprehensively gather and discuss scientific findings covering the role of *Blastocystis* sp. in IBS *via* gut microbiota shifting.

Keywords: irritable bowel syndrome, gut microbiota, *Blastocystis* sp., dysbiosis, post-infectious-IBS

OPEN ACCESS

Edited by:
Md. Robiul Karim,
Bangabandhu Sheikh Mujibur Rahman Agricultural University, Bangladesh

Reviewed by:
Ali Rostami,
Babol University of Medical Sciences, Iran
Iraj Sharifi,
Kerman University of Medical Sciences, Iran

***Correspondence:**
Hamed Mirjalali
hamed_mirjalali@hotmail.com;
hamedmirjalali@sbmu.ac.ir
Elnaz Sadat Mirsamadi
elnaz_mir62@yahoo.com

Specialty section:
This article was submitted to Gastroenterology, a section of the journal Frontiers in Medicine

Received: *07 March 2022*
Accepted: *09 May 2022*
Published: *20 June 2022*

Citation:
Olyaiee A, Sadeghi A, Yadegar A, Mirsamadi ES and Mirjalali H (2022) Gut Microbiota Shifting in Irritable Bowel Syndrome: The Mysterious Role of Blastocystis sp. Front. Med. 9:890127. doi: 10.3389/fmed.2022.890127

INTRODUCTION

Irritable bowel syndrome (IBS) is a chronic disorder, which is known by abdominal pain and abnormal defection. IBS seems to be a gut-brain axis-related disease; therefore, it is also called a functional gastrointestinal (GI) disorder (1, 2). IBS is a commonly reported disorder in clinical practices, affecting 10–20% of the world's population (3).

IBS could be asymptomatic; however, this disease is characterized by symptoms such as abdominal pain, variable bowel habits, and bloating (1). IBS is a multifactorial disorder. Even though its causative agent is not clear; gut microbiota disturbance appears to play an important role in this disorder (4). Early studies have documented the role of microbial gastroenteritis [post-infectious IBS (PI-IBS)] (5, 6) and overuse of broad-spectrum antibiotics in the development of IBS (7). Studies on the gut microbiota in patients with IBS suggest that microbial dysbiosis may increase the severity and duration of IBS symptoms. Accordingly, it was documented that changes in the gut microbiota composition play a critical role in establishing, developing, and flaring the symptoms of IBS (8). Nevertheless, the manipulation of the intestinal microbiota can be considered a new treatment method for patients with IBS. For example, fecal microbiota transplantation (FMT) reduced inflammation and symptoms in people with IBS and could be regarded as a treatment strategy (9).

In addition to the critical role of bacteria in the development of IBS, the high prevalence of some protozoa, such as *Blastocystis* sp., has highlighted the putative role of this protist in the development of IBS. Although a bilateral correlation between the presence of *Blastocystis* sp. and the gut microbiota composition has been reported in many studies, it is not clear whether the gut conditions and gut microbiota perturbation lead to higher colonization of *Blastocystis* sp. in the gut or colonization of *Blastocystis* sp. may lead to dysbiosis (10, 11).

PARASITES AND IBS

It has been suggested that microbial infections may lead to a mild inflammation through the intestine and the development of IBS. Although the results contradict (12), the emerging role of intestinal parasites in the development of IBS is now being investigated (13). Intestinal parasites can increase the gut permeability and contact of lumen antigens with the lower layers of the intestine, provoking immune responses, and developing chronic inflammation (14, 15).

Accordingly, a higher prevalence of some protozoa (*Giardia*, *Blastocystis*, and *Cryptosporidium*) was recorded among the patients with IBS compared to healthy controls (16). The correlation between previous infection with *Giardia* and an increased risk of IBS was strongly suggested by retrospective studies. In this regard, in a retrospective cohort study performed by Dormond et al. (17), which was carried out on military personnel, the risk of developing chronic GI disorders, such as IBS, was assessed in those with documented giardiasis and the findings represented an increased risk of IBS in *Giardia*-infected personnel (17). Nakao et al. (18), in a large retrospective study, which employed the 2006–2010 MarketScan commercial insurance database, showed that despite considering confounding factors, such as anxiety, depression, and healthcare utilization, giardiasis increased the risk of subsequent IBS (18). Although the mechanism beyond the role of giardiasis in increasing the risk of IBS is not clear, destruction of the intestinal barrier unity during colonization of *G. lamblia* appears to be an important factor. For example, it was demonstrated that infection by *Giardia* may lead to gut barrier dysfunctions *via* downregulation of tight junctions, such as claudin-1, and induction of apoptosis in epithelial cells throughout the intestine (19).

There is evidence speculating the role of early-age infection by *Cryptosporidium* in the development of IBS in adolescence, *via* increased jejunal sensitivity to bloating (20). In addition, the intestinal stage of a nematode worm, *Trichinella spiralis*, seems to provoke a mild inflammation over the gut epithelial cell, which can lead to visceral sensitivity and changes in intestinal motility (21–23). It was shown that transient mucosal inflammation in Swiss mice during the acute phase of infection by *T. spiralis* can lead to an alteration in neuromuscular functions, even after the resolution of *Trichinella*-caused inflammation (21). These findings were then supported by Venkova et al. (23), who demonstrated jejunal inflammation due to *T. spiralis*, which induced long-term changes in muscle contractility and enteric neurotransmission, even after recovery from mucosal inflammation (23). Eventually, it was demonstrated that during the GI phase of trichinosis, *T. spiralis* can induce a long-term remodeling of epithelial functions (22). Therefore, the persistent inflammation and neuromuscular dysfunctions during the intestinal stage of trichinosis, seem to increase the risk of IBS development, particularly in those who are susceptible.

Dientamoeba fragilis is an intestinal protozoan, which, together with *Blastocystis* sp., are the most prevalent protozoa reported in patients with IBS (24–26). The probable correlation between *D. fragilis* and IBS was first reported by Borody et al. (27) who showed that eradication of the protozoan led to amelioration of IBS-like symptoms. However, further investigations on the correlation between *D. fragilis* and IBS have reported controversial results. For example, Yakoob et al. (28) demonstrated a higher prevalence of *D. fragilis* in patients with IBS compared to healthy controls. Engsbro et al. (25) reported that 35–41% of patients with IBS carried out *D. fragilis* in the Danish population, and Ibrahim et al. (29) supported previous studies and documented a higher prevalence of *D. fragilis* in patients with IBS compared to control subjects. A case of PI-IBS due to *D. fragilis* in a patient who traveled to Mexico points out the probable role of this protozoan in the development of IBS, as well (30). However, in contrast to these studies, Engsbro et al. (31) analyzed the response to anti-*D. fragilis* treatment in 25 patients with IBS who carried the protozoan and showed the lack of correlation between microbiological response to treatment and clinical manifestations of patients with IBS.

THE HIGH PREVALENCE OF *BLASTOCYSTIS* SP. IN PATIENTS WITH IBS

Blastocystis sp. is frequently reported from patients with IBS, and numerous studies have suggested a correlation between carrying this protist and IBS; however, it is not clear whether either *Blastocystis* sp. leads to IBS/IBS-like symptoms or perturbed conditions of the GI tract provide a favorable niche for colonization of *Blastocystis* sp. One of the first studies that examined a probable parasite as a causative agent of IBS was performed by Yakoob et al. (32), who reported a statistically significant higher prevalence of *Blastocystis* sp. in patients with IBS [46% (44 of 95)] compared to the controls [7% (4 of 55)]. Surangsrirat et al. (33), in a case–control study in Thailand, documented a higher frequency of *Blastocystis* sp. in patients with IBS (16.7%) compared to the control group (10%), although this difference was not statistically significant. In addition, Das et al. (34) demonstrated that the prevalence of *Blastocystis* sp. was three times higher than that reported in healthy subjects.

The close frequency rate of *Blastocystis* sp. in patients with IBS and control groups in many studies and a greater prevalence rate of *Blastocystis* sp. in control groups compared to patients with IBS have obscured an established linkage between the presence of this protist and the development of IBS (35–37).

The presence of correlation between genetic lineages of *Blastocystis* sp. and the development of IBS has also been

evaluated. Although a correlation between the presence of ST1 (38) and ST3 (39) with IBS was suggested, most of the studies failed to associate the presence of a certain subtype with IBS development. In this regard, Pena et al. (40) analyzed the subtype distribution of *Blastocystis* sp. in patients with IBS compared to healthy controls that the results showed the presence of ST1 and ST2 in both groups, while ST3 and ST4 were only characterized by healthy controls and patients with IBS, respectively. The lack of linkage between certain subtypes and IBS was observed in Indian subjects, where ST3 was the dominant subtype in both IBS and control groups followed by ST1 (34). Subtypes 1 and 3 were also reported to be the major genetic lineages in IBS and control subjects in other studies (41–43).

The expression of certain enzymes in *Blastocystis* sp. isolated from patients with IBS was also evaluated. In this regard, Nagel et al. (44) indicated the higher presence of a *Blastocystis* sp. protein (probably a cysteine protease) in subjects with IBS compared to healthy controls. They also claimed that some single nucleotide polymorphisms (SNPs) in IL-8 and IL-10 probably affect the relative risk of IBS development in individuals who carry *Blastocystis* sp. (45). Although the effects of genetic polymorphisms through the IBS-related signature regions of IL-6, IL-8, and IL-10 might be different in various ethnic groups, these SNPs could increase the risk of IBS development (46, 47). In this line, a recently published study proposed significantly higher serum levels of IL-6, IL-8, IL-10, IFN-γ, and TNF-α in patients with IBS who colonized with *Blastocystis* sp., proposing the critical effects of *Blastocystis* sp., in IBS development (48) *via* modulation of IBS-related cytokines (49).

Taken together, although colonization of a certain subtype of *Blastocystis* sp. does not seem to be correlated with IBS, it was suggested that there are significant differences in the protease activity of different subtypes of *Blastocystis* sp. (50). Importantly, proteases released by *Blastocystis* sp. can disrupt the epithelial barrier and actin filaments, increase gut permeability, and subsequently develop IBS (51–53). Moreover, a most recently published study suggested that *Blastocystis* sp. ST3 can modulate the expression levels of microRNAs involved in the gut barrier integrity, and claudin-7 (54). Notably, claudin-7 is categorized among pre-sealing tight junction proteins and plays a critical role in reducing the permeability of the gut (55).

A BRIEF LOOK AT THE GUT MICROBIOTA

The microbiota is comprised of the microbial community of the human body, which is made up of a variety of microorganisms, including bacteria, viruses, fungi, and protozoa. The gut microbiota consists of between 10 and 100 trillion cells, which are ∼10 times the total cells of a human (56). The gut microbiome has the highest number of microbial communities. The number of microbial genes in the gut is estimated to be 150 times higher than the genes of human origin (57). Current documentations demonstrated four bacterial phyla, including Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria, are the core of the gut microbiota of which Firmicutes and Bacteroidetes are predominant (58–60).

The gut microbiota composition is linked to a couple of intestinal and extra-intestinal disorders (61–64). Changes in the GI microbiota (is known as dysbiosis) can affect the immune responses, metabolism, and intestinal permeability, resulting in a pre-inflammatory state. Such changes can disrupt the functions of the host's immunity and metabolic systems, which may lead to diseases, such as diabetes, obesity, GI, neurological, and autoimmune disorders (61, 63, 65, 66). A number of studies have demonstrated a link between changes in the gut microbiota and incidences of gut-related diseases, such as obesity, non-alcoholic steatohepatitis (NASH), IBS, inflammatory bowel disease (IBD), celiac disease, and GI neoplasms (61, 66–68).

THE GUT MICROBIOTA IN IBS

The correlation between gut microbiota dysbiosis and IBS conditions is well-established (69, 70). A correlation between the severity of IBS and a gut microbiota signature has been demonstrated (71). PI-IBS, small intestine bacterial overgrowth (SIBO), stress, antibiotics, diet, and early childhood experiences shape the gut microbiota and affect the incidence rate of IBS (72).

A gut microbiota analysis suggested a doubled ratio of Firmicutes to Bacteroidetes, an increased number of *Dorea*, *Ruminococcus*, and *Clostridium* spp., and a decreased number of *Faecalibacterium* spp. in patients with IBS compared to healthy controls (69). Tap et al. (71) reported an association between the gut microbiota composition and the severity of IBS. They showed that enterotypes *Prevotella* and *Bacteroides* represented the lowest and highest proportion in patients with severe IBS, respectively. In addition, it was claimed that the highest and lowest proportion of *Bacteroides* were observed in IBS-D and IBS-C, respectively (71). These results were then confirmed by Zhuang et al. (73) who documented the Bacteroidetes in stool samples of patients with IBS-D, and suggested an association between the gut microbiota composition with the pathogenicity of IBS (73). IBS has four types, which are characterized based on the stool formation and the number of defecation, including IBS-C with constipation; IBS-D with diarrhea; IBS-M, which has intermittent bowel pattern with a mix of diarrhea and constipation; and IBS-U, which is not easily classified into any of the mentioned groups (74). Metagenomics studies suggest that the gut microbiota may present different patterns according to the types of IBS. The results of a quantitative real-time PCR, which was employed to amplify the 16S rRNA gene of the gut bacteria, showed that the number of bacteria, such as *R. productus*, *C. coccoides*, *Villonella*, *Tetiotamicron*, *Pseudomonas aeruginosa*, and Gram-negative bacteria in patients with IBS was higher, but the number of *Lactobacillus* was lower than healthy individuals. In addition, the number of *Violinella* species in patients with IBS-C and *P. aeruginosa* in people with IBS-C and IBS-D were higher than in healthy individuals (75).

The 16S rRNA gene sequencing revealed that although the gut microbiota diversity was similar between patients with IBS and healthy controls, the richness of bacteria in patients with IBS-D was lower than in other groups, while a significant increase in Proteobacteria and decrease in Firmicutes, Fusobacteria,

and Actinobacteria were observed in patients with IBS-D (76). Apart from the changes in the diversity and richness of the gut microbiota, the overgrowth of the bacterial community throughout the small intestine is thought to be associated with IBS (4). A systematic review by Pittayanon et al. (77) on 24 studies revealed that Enterobacteriaceae (phylum Proteobacteria), family Lactobacillaceae, and genus *Bacteroides* were increased, and uncultured Clostridiales I, genus *Faecalibacterium* (e.g., *F. prausnitzii*), and genus *Bifidobacterium* were decreased in patients with IBS compared to healthy controls. Nevertheless, the presence of a microbiome signature, how IBS alters the gut microbiota compositions, and the role of diet changes on the gut microbiota alterations in patients with IBS are the main unclear issues that need to be investigated (**Table 1**).

THE GUT MICROBIOTA IN IBS AND IMMUNE SYSTEM

There is growing evidence linking inflammation with IBS. Inflammation may lead to changes in smooth muscles and GI nerves, which are resulted in GI dysfunctions (86, 87). The presence and the number of mast cells, release of histamine and tryptase, distance of intestinal nerve to mast cells in patients with IBS characterized by Room II, and abdominal pain were compared to healthy controls that the infiltration of mast cells and release of their contents in proximity to mucosal innervation were probably correlated with IBS and abdominal pain (88).

The correlation between low level of inflammation and IBS manifestations was suggested in a study by Ohman et al. (89) who showed an increased frequency of blood T cells expressing CD69 and integrin b7/HLA-DR. They concluded that T-cell activation supports low-grade inflammation and symptom generation in patients with IBS (89). This finding was later supported by Nasser et al. (90), who showed an immune activation of CD4+ T cell derived from patients with IBS-D, while it was not correlated with physiological stress. However, they suggested that immune activation is could be a trigger of or a parallel phenomenon with IBS (90). The higher number and the activation of mucosal B lymphocytes and plasma cells, together with an increased number of mast cells in the mucosal jejunal biopsy of patients with IBS-D, probably contribute to the presentation of the disorder (91). Therefore, it seems that a mild inflammation due to enhanced humoral and innate immunity throughout the intestine could be correlated with IBS (91, 92). Importantly, amelioration of the clinical manifestations of IBS-D *via* activation of mast cells and modulating of the intestinal innate immunity followed by oral prescription of disodium cromoglycate (DSCG) confirmed the hypothesis of correlation between mild inflammation and IBS development (93).

The role of gut microbiota in the arrangement of the immune responses in IBS is still investigating; however, it seems that microbe-association pattern recognition plays a determinative role in orchestrating the immune responses (94). In this regard, the role of toll-like receptors (TLRs) as cross-road between gut microbiota, immune responses, and IBS, has been highlighted. Among TLRs, TLR4 seems to be more involved in the development of IBS. TLR4 interacts with lipopolysaccharide (LPS) pattern, which is the most outer surface component of almost all Gram-negative bacteria (95, 96). It was demonstrated that stimulation of TLR4 by LPS can lead to motivation of the enteric nervous system and motility of the intestine (97). This finding was further investigated and supported by the comparison of the expression of TLRs in biopsy samples of patients with IBS and healthy controls in which the results implied a significant elevation of TLR4 in patients with IBS (98). Furthermore, Belmonte et al. (99) evidenced that the levels of TLR may be different based on the IBS subtypes. In this regard, they reported a significant upregulation of TLR2 and TLR4 in patients with IBS-M together with elevation of IL-8 and IL-1β (99). Recently, Jalanka et al. (100) analyzed the correlation of the gene expression of TLR4 and correlated receptors in patients with IBS and supported the probable role of a low inflammation due to bacteria in the intestine of patients with IBS. Therefore, it seems that a change in the gut microbiota composition may arrange a chronic inflammation and subsequent IBS.

THE CORRELATION BETWEEN *BLASTOCYSTIS* SP. AND THE GUT MICROBIOTA IN PATIENTS WITH IBS

Many studies demonstrated the effects of colonization of *Blastocystis* sp. on the gut microbiota, in both composition and richness. It was suggested that the presence of *Blastocystis* sp. reduced a Firmicutes/Bacteroidetes ratio (F/B ratio) in two cohort studies, FACSA and UNEME, which were performed in healthy people and individuals with metabolic disorders in Mexico (101). The mean proportions of *Faecalibacterium* spp. and Ruminococcaceae in the *Blastocystis* sp.-positive group, and *Enterococcus* spp. in the *Blastocystis* sp.-negative group were abundant in Korean populations (102). The high richness of *Faecalibacterium*, *Prevotella*, Ruminococcaceae UCG-002, Muribaculaceae, Rikenellaceae, Acidaminococcaceae, *Phascolarctobacterium*, and Ruminococcaceae UCG-005 in individuals who carry *Blastocystis* sp., and *E. hirae*, *E. faecalis*, *E. durans*, Enterococcaceae, Lactobacillales, and Bacilli in subjects who were negative for *Blastocystis* sp. was observed (102). Importantly, this study concluded that the presence of *Blastocystis* sp. is an indicator of healthy gut microbiota (102). Most recently, it was shown that *Blastocystis* sp. ST4 inhibited the growth of *B. vulgatus*, which suggests the protective role of *Blastocystis* sp. ST4 in the gut barrier integrity from damage due to the bacteria (103). Later, an altered gut microbial composition was documented in normal healthy mice and $Rag1^{-/-}$ mice colonized by *Blastocystis* sp. ST4, mainly by an increased proportion of Clostridia vadinBB60 group and Lachnospiraceae NK4A136 group, respectively (104). These results confirmed the protective role of *Blastocystis* sp. ST4 in the modulation of the gut microbiota to reduce inflammation (104). *Blastocystis* sp. was reported to significantly increase alpha diversity in carriers. Accordingly, the presence of *Blastocystis* sp. was associated with enriched Firmicutes and Bacteroidetes, and the genera *Prevotella*, *Faecalibacterium*, *Flavonifractor*, *Clostridium*, *Succinivibrio*, and

TABLE 1 | The gut microbiota changes in patients with IBS.

No.	Sample type	Age (average)	Method	Number of patients	Number of healthy	IBS patients		References
						Increase	Decrease	
1	Stool	NP	qPCR	47	30	*Lactobacillus* *Ruminococcus* *Veillonella* *Bacteroides* *Pseudomonas* *Clostridium*	*Bifidobacterium* *Entrococcus*	(75)
2	Stool	Adult (28–59)	16S rRNA gene sequencing	44	47	Bacteroidetes	Proteobacteria Firmicutes	(78)
3	Stool	Adult (22–66)	qPCR, Microarray Analysis	62	46	*Streptococcus* *Clostridium* *Papillibacter* *Ruminococcus* *Sporobacter* *Dialister* *Peptococcus* *Blautia* *Butyrivibrio* *Dorea* *Roseburia* *Lachnospira*	Bifidobacteria *Alistipes* Bacteroidetes *Odoribacter* Parabacteroides *Prevotella* *Faecalibacterium*	(69)
4	Duodenal mucosa and lumen, Rectal mucosa and lumen	Adult	16S rRNA gene sequencing	74	20	*Bacteroides* *Prevotella* *Oscillospira*	–	(79)
5	stool	Adult (37–60)	16S rRNA gene sequencing	20	18	*Enterobacter* *Streptococcus* *Fusobacterium* *Rothia*	*Roseburia* *Faecalibacterium*	(80)
6	Stool, Mucosal Samples	Adults (18–65)	16S rRNA gene sequencing, qPCR	110	39	Bacteroidetes Clostridiales *Lachnospira* *Ruminococcus*	*Faecalibacterium* *Prevotella* Firmicutes *Blautia* *Coprococcus*	(71)
7	Stool	Adult	16S rRNA gene sequencing	27	13	Bacteroidetes Fusobacteria Faecalibacter	Firmicutes Proteobacteria *Lachnospira* *Ruminococcus* *Lactobacillus*	(73)
8	Stool	Adult	16S rRNA gene sequencing	30	30	Proteobacteria Enterobacter Bacteroidetes *Streptococcus* *Lactobacillus* *Escherichia coli* *Proteobacteria* *Enterobacter*	Firmicutes Fusobacteria Actinobacteria *Clostridia* *Ruminococcus* *Faecalibacterium* *B. pseudocatenulatum*	(76)
9	Stool	Adult	Metagenomics gene-targeted approach	3	8	*Dialister* *Faecalibacterium* Alcaligenaceae	–	(81)
10	Stool	Adult (27–46)	16S rRNA gene sequencing	19	16	*Ruminococcus*	–	(82)
11	Stool	Adult/child	16S rRNA gene sequencing, qPCR	74	21	*Escherichia/Shigella* *Aeromonas*	*Actinobacteria* *Citrobacter* *Microvirgula*	(83)
12	Stool, Sigmoid biopsy	Adult/child	454 pyrosequencing	40	20	Bacteroidetes *Prevotella*	Lachnospiraceae incertae sedis, *Coprococcus,*	(84)

(Continued)

TABLE 1 | Continued

No.	Sample type	Age (average)	Method	Number of patients	Number of healthy	IBS patients		References
						Increase	Decrease	
							Clostridium XI, *Odoribacter, Butyricimonas, Alistipes, Blautia*	
13	Stool	Adult	16S rRNA gene sequencing	16	21	Clostridiales Acidimicrobidae Lachnospiraceae *Clostridiales*	Actinobacteria Rhodospirillales Burkholderiales Cyanobacteria Sphingomonadaceae	(85)

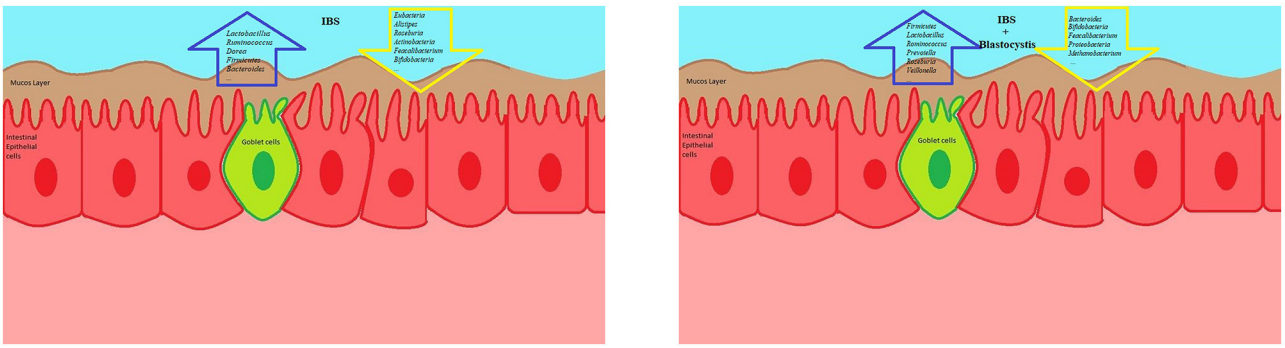

FIGURE 1 | The schematic view presenting a correlation between *Blastocystis* sp. and certain microbiota signatures. The presence of *Blastocystis* sp. has not been linked with specific signature of gut microbiota. Although this protist seems to a healthy indicator for the gut microbiota, *Blastocystis* sp. can reduce a Firmicutes/Bacteroidetes ratio and protective bacteria. In addition, the low and high presence of *Blastocystis* sp. in IBD and IBS, respectively, might be related to the differences in the gut microbiota composition between IBD and IBS.

Oscillibacter, whereas Proteobacteria and the genera *Escherichia*, *Bacteroides*, *Klebsiella*, and *Pseudomonas* were enriched in *Blastocystis* sp.-negative group (105).

In contrast, a positive correlation between the presence of *Blastocystis* sp. and *C. difficile* was suggested in patients with IBD, which was also correlated with the number of defecation in these patients (10). Furthermore, Nourrisson et al. (106) investigated the gut microbiota in four classes of IBS and concluded that the presence of *Blastocystis* sp. was significantly correlated with an increase of *Lactobacilli* and a decrease of *Bifidobacterium* sp. and *F. prausnitzii* in male patients. Therefore, they suggested that *Blastocystis* sp. colonization may lead to a decrease in protective bacteria (106). To clear the role of *Blastocystis* sp., in shifting the gut conditions or gut microbiota composition in IBS, an experimental study was performed by Defaye et al. (107) in rats. Accordingly, they orally inoculated *Blastocystis* sp. ST4 in rats and evaluated the gut microbiota, inflammation, behavior, and short-chain fatty acid (SCFA). Interestingly, their findings showed that the presence of *Blastocystis* sp. was resulted in non-inflammatory colonic hypersensitivity with increased serine protease activity, anxiety- and depressive-like behaviors, the relative abundance of *Oscillospira* with a decrease in *Clostridium*, and lower levels of SCFAs (107), all of which highlight the role of *Blastocystis* sp. in the development of IBS and its symptoms, as well as microbiota shifting in patients with IBS (107).

Recently, the eukaryome and prokaryome profiles of patients with IBS-C showed that the colonization of *Blastocystis* sp. not only induces changes in prokaryotic microbiota of the gut, particularly Tenericutes phylum and Ruminococcaceae family, but also this protist may disturb eukaryome population, particularly fungi, in patients with IBS (108). Importantly, gut mycobiome seems to play an important role in the development, presentation of symptoms, and response to treatment in patients with GI disorders (109–111). Nevertheless, the role of *Blastocystis* sp. in the gut microbiota changes is controversial, and there is evidence of a neutral role of this protist in the gut microbiota composition in patients with IBS (74). The controversial correlation between the gut microbiota composition and colonization of *Blastocystis* sp. (112) is thought to be related to antibiotic consumption (113). Remarkably, antibiotics can alter the gut microbiota composition and change the gut lumen from a favorable niche for *Blastocystis* sp. colonization toward an inimical condition for the protist (**Figure 1** and **Table 2**).

TABLE 2 | The gut microbiota changes in patients with IBS carrying *Blastocystis* sp.

No.	Sample types	Age	Methods	Number of patients	Number of healthy	IBS patients		*Blastocystis*-positive IBS patients		*Blastocystis*-positive control subjects		References
						Increase	Decrease	Increase	Decrease	Increase	Decrease	
1	Stool	Adult	16S rRNA	40 (24 *Blastocystis* positive and 14 *Blastocystis* negative)	57 (42 *Blastocystis* positive and 15 *Blastocystis* negative)	Actinobacteria, Cyanobacteria, Elusimicrobia, Firmicutes, Fusobacteria, Proteobacteria, Methanobacteria, *Streptococcus, Clostridia,* Lachnospiraceae, Alphaproteobacteria, *Blautia*	Bacteroidetes, *Veillonella, Dialister, Catenibacter, Butyricimonas, Olsenella*	Bacteroidetes, Cyanobacteria, Firmicutes, Fusobacteria	Actinobacteria, Elusimicrobia, Proteobacteria,	–	–	(74)
2	Stool	Adult	qPCR 16S rRNA	56	56	Bacteroidetes, Firmicutes	–	–	Bifidobacteria, Faecalibacter, *Lactobacillus*	*Lactobacillus*	Bifidobacteria	(106)
3	Stool	Adult	16S rRNA qPCR	35	23	Proteobacteria Bacteroidetes Tenericutes	Firmicutes	Actinobacteria, Firmicutes	Bacteroidetes Proteobacteria	*Ruminococcus* *Bacteroidetes* *Tenericutes*	Firmicutes Actinobacteria Bacteroidetes Proteobacteria	(108)

CONCLUDING REMARKS

Blastocystis sp. is a prevalent protist, which is reported from apparently healthy subjects, as well as individuals with a variety of GI disorders. Although the presence of *Blastocystis* sp. has not been linked with certain symptoms or disorders, this protist is reported in high prevalence rate in some diseases, such as IBS. IBS conditions seem to provide a favorable niche for colonization of *Blastocystis* sp. in the intestine. It is not clear how *Blastocystis* sp. communicates with the gut microbiota in healthy and disease conditions, particularly in patients with IBD and IBS. Some studies suggested that *Blastocystis* sp. is a healthy gut indicator, while the high prevalence of this protist in IBS proposes a correlation between improper gut conditions and *Blastocystis* sp. colonization. Nevertheless, recent studies have indicated a correlation between *Blastocystis* sp. and gut microbiota. It is not clear that either *Blastocystis* sp. may manipulate the gut microbiota composition or an altered gut microbiota in IBS may provide favorable conditions for colonization of *Blastocystis* sp.

Gut permeability is a key point of IBS development. *Blastocystis* sp. discharges a number of proteins, particularly a broad spectrum of proteases, which affect the gut permeability and tight junctions. Although limited data, the secretion levels and types of proteases and the effects of proteases on the human cells are supposed to be different in *Blastocystis* sp. strains. Therefore, the study of proteases, particularly cysteine protease, derived from different isolates and subtypes on the gut permeability and tight junction proteins provides interesting data. There is no documented study investigating the role of extracellular vesicles (EVs) discharged from either *Blastocystis* sp. or *Blastocystis* sp.-affected host cell on the gut permeability. Indeed, a cross-talk between *Blastocystis* sp. and gut microbiota *via* EVs may play a role in the successful colonization of the protist or the gut microbiota composition of the gut. EVs play an important role in cross-talk between microorganisms and the study of EVs (released from *Blastocystis* sp., gut microbiota, and/or host cells) would be interesting. However, researches on *Blastocystis* sp. is at the beginning stages and more studies are needed to be performed.

ETHICS STATEMENT

This study was approved by the ethical standards (IR.IAU.SRB.REC.1400.241) released by Ethical Review Committees of Islamic Azad University Science and Research Branch, Tehran, Iran.

AUTHOR CONTRIBUTIONS

HM and ESM: conceived and designed. AO, HM, ESM, and AY: data gathering and literature review. AO and HM: writing the manuscript. AS: reviewing and editing the manuscript. All authors read and approved the final version of the manuscript.

ACKNOWLEDGMENTS

The authors thank all members of the Foodborne and Waterborne Diseases Research Center for their support.

REFERENCES

1. Ford AC, Lacy BE, Talley NJ. Irritable bowel syndrome. *N Engl J Med.* (2017) 376:2566–78. doi: 10.1056/NEJMra1607547
2. Lacy BE, Pimentel M, Brenner DM, Chey WD, Keefer LA, Long MD, et al. ACG Clinical guideline: management of irritable bowel syndrome. *Am J Gastroenterol.* (2021) 116:17–44. doi: 10.14309/ajg.0000000000001036
3. Dale HF, Lied GA. Gut microbiota and therapeutic approaches for dysbiosis in irritable bowel syndrome: recent developments and future perspectives. *Turk J Med Sci.* (2020) 50:1632–41. doi: 10.3906/sag-2002-57
4. Bennet SM, Ohman L, Simren M. Gut microbiota as potential orchestrators of irritable bowel syndrome. *Gut Liver.* (2015) 9:318–31. doi: 10.5009/gnl14344
5. Berumen A, Lennon R, Breen-Lyles M, Griffith J, Patel R, Boxrud D, et al. Characteristics and risk factors of post-infection irritable bowel syndrome after *Campylobacter* enteritis. *Clin Gastroenterol Hepatol.* (2021) 19:1855–63.e1851. doi: 10.1016/j.cgh.2020.07.033
6. Sung J, Morales W, Kim G, Pokkunuri V, Weitsman S, Rooks E, et al. Effect of repeated *Campylobacter jejuni* infection on gut flora and mucosal defense in a rat model of post infectious functional and microbial bowel changes. *Neurogastroenterol Motil.* (2013) 25:529–37. doi: 10.1111/nmo.12118
7. Ianiro G, Tilg H, Gasbarrini A. Antibiotics as deep modulators of gut microbiota: between good and evil. *Gut.* (2016) 65:1906–15. doi: 10.1136/gutjnl-2016-312297
8. Fagoonee S, Pellicano R. Does the microbiota play a pivotal role in the pathogenesis of irritable bowel syndrome? *J Clin Med.* (2019) 8:1808. doi: 10.3390/jcm8111808
9. Johnsen PH, Hilpüsch F, Valle PC, Goll R. The effect of fecal microbiota transplantation on IBS related quality of life and fatigue in moderate to severe non-constipated irritable bowel: secondary endpoints of a double blind, randomized, placebo-controlled trial. *EBioMed.* (2020) 51:102562. doi: 10.1016/j.ebiom.2019.11.023
10. Azimirad M, Gol SMA, Javanmard E, Mirjalali H, Yadegar A, Aghdaei HA, et al. *Blastocystis* and *Clostridioides difficile*: evidence for a synergistic role in colonization among IBD patients with emphasis on ulcerative colitis. *Turk J Gastroenterol.* (2021) 32:500–7. doi: 10.5152/tjg.2021.19644
11. Mirjalali H, Abbasi MR, Naderi N, Hasani Z, Mirsamadi ES, Stensvold CR, et al. Distribution and phylogenetic analysis of *Blastocystis* sp. subtypes isolated from IBD patients and healthy individuals in Iran. *Europ J Clin Microbiol Infect Dis.* (2017) 36:2335–42. doi: 10.1007/s10096-017-3065-x
12. Morgan DR, Benshoff M, Cáceres M, Becker-Dreps S, Cortes L, Martin CF, et al. Irritable bowel syndrome and gastrointestinal parasite infection in a developing nation environment. *Gastroenterol Res Pract.* (2012) 2012:343812. doi: 10.1155/2012/343812
13. Lee YY, Annamalai C, Rao SSC. Post-infectious irritable bowel syndrome. *Curr Gastroenterol Rep.* (2017) 19:56. doi: 10.1007/s11894-017-0595-4
14. Beatty JK, Bhargava A, Buret AG. Post-infectious irritable bowel syndrome: mechanistic insights into chronic disturbances following enteric infection. *World J Gastroenterol.* (2014) 20:3976–85. doi: 10.3748/wjg.v20.i14.3976
15. Motomura Y, Khan WI, El-Sharkawy RT, Verma-Gandhu M, Grencis RK, Collins SM. Mechanisms underlying gut dysfunction in a murine model of chronic parasitic infection. *Am J Physiol Gastrointestinal Liver Physiol.* (2010) 299:G1354–60. doi: 10.1152/ajpgi.00324.2010

16. Jadallah KA, Nimri LF, Ghanem RA. Protozoan parasites in irritable bowel syndrome: a case-control study. *World J Gastrointestinal Pharmacol Therap.* (2017) 8:201–7. doi: 10.4292/wjgpt.v8.i4.201
17. Dormond M, Gutierrez RL, Porter CK. *Giardia lamblia* infection increases risk of chronic gastrointestinal disorders. *Trop Dis Travel Med Vaccin.* (2016) 2:17. doi: 10.1186/s40794-016-0030-0
18. Nakao JH, Collier SA, Gargano JW. Giardiasis and subsequent irritable bowel syndrome: a longitudinal cohort study using health insurance data. *J Infect Dis.* (2017) 215:798–805. doi: 10.1093/infdis/jiw621
19. Troeger H, Epple HJ, Schneider T, Wahnschaffe U, Ullrich R, Burchard GD, et al. Effect of chronic *Giardia lamblia* infection on epithelial transport and barrier function in human duodenum. *Gut.* (2007) 56:328–35. doi: 10.1136/gut.2006.100198
20. Marion R, Baishanbo A, Gargala G, François A, Ducrotté P, Duclos C, et al. Transient neonatal *Cryptosporidium parvum* infection triggers long-term jejunal hypersensitivity to distension in immunocompetent rats. *Infect Immun.* (2006) 74:4387–9. doi: 10.1128/IAI.02055-05
21. Barbara G, Vallance BA, Collins SM. Persistent intestinal neuromuscular dysfunction after acute nematode infection in mice. *Gastroenterology.* (1997) 113:1224–32. doi: 10.1053/gast.1997.v113.pm9322517
22. Venkova K, Greenwood-van Meerveld B. Long-lasting changes in small intestinal transport following the recovery from *Trichinella spiralis* infection. *Neurogastroenterol Motil.* (2006) 18:234–42. doi: 10.1111/j.1365-2982.2005.00753.x
23. Venkova K, Palmer JM, Greenwood-Van Meerveld B. Nematode-induced jejunal inflammation in the ferret causes long-term changes in excitatory neuromuscular responses. *J Pharmacol Exp Therap.* (1999) 290:96–103.
24. Abedi SH, Fazlzadeh A, Mollalo A, Sartip B, Mahjour S, Bahadory S, et al. The neglected role of *Blastocystis* sp. and *Giardia lamblia* in development of irritable bowel syndrome: a systematic review and meta-analysis. *Microbial Pathog.* (2022) 162:105215. doi: 10.1016/j.micpath.2021.105215
25. Engsbro AL, Stensvold CR, Vedel Nielsen H, Bytzer P. Prevalence, incidence, and risk factors of intestinal parasites in Danish primary care patients with irritable bowel syndrome. *Scand J Infect Dis.* (2014) 46:204–9. doi: 10.3109/00365548.2013.861609
26. Rostami A, Riahi SM, Haghighi A, Saber V, Armon B, Seyyedtabaei SJ. The role of *Blastocystis* sp. and *Dientamoeba fragilis* in irritable bowel syndrome: a systematic review and meta-analysis. *Parasitol Res.* (2017) 116:2361–71. doi: 10.1007/s00436-017-5535-6
27. Borody T, Warren E, Wettstein A, Robertson G, Recabarren P, Fontella A, et al. Eradication of *Dientamoeba fragilis* can resolve IBS-like symptoms. *J Gastroenterol Hepatol.* (2002) 17:A103.
28. Yakoob J, Jafri W, Beg MA, Abbas Z, Naz S, Islam M, et al. *Blastocystis hominis* and *Dientamoeba fragilis* in patients fulfilling irritable bowel syndrome criteria. *Parasitol Res.* (2010) 107:679–84. doi: 10.1007/s00436-010-1918-7
29. Ibrahim AN, Al-Ashkar AM, Nazeer JT. Additional glance on the role of *Dientamoeba fragilis* & *Blastocystis hominis* in patients with irritable bowel syndrome. *Iranian J Parasitol.* (2018) 13:100–7.
30. Ali S, Khetpal N, Khan MT, Rasheed M, Asad-Ur-Rahman F, Echeverria-Beltran K. A mexican honeymoon marred by gastrointestinal upset: a case of *Dientamoeba fragilis* causing post-infectious irritable bowel syndrome. *Cureus.* (2017) 9:e1992. doi: 10.7759/cureus.1992
31. Engsbro AL, Stensvold CR, Nielsen HV, Bytzer P. Treatment of *Dientamoeba fragilis* in patients with irritable bowel syndrome. *Am J Trop Med Hyg.* (2012) 87:1046–52. doi: 10.4269/ajtmh.2012.11-0761
32. Yakoob J, Jafri W, Jafri N, Khan R, Islam M, Beg MA, et al. Irritable bowel syndrome: in search of an etiology: role of *Blastocystis hominis*. *Am J Trop Med Hyg.* (2004) 70:383–5. doi: 10.4269/ajtmh.2004.70.383
33. Surangsrirat S, Thamrongwittawatpong L, Piyaniran W, Naaglor T, Khoprasert C, Taamasri P, et al. Assessment of the association between *Blastocystis* infection and irritable bowel syndrome. *J Med AssocThailand.* (2010) 93(Suppl. 6):S119–24.
34. Das R, Khalil S, Mirdha BR, Makharia GK, Dattagupta S, Chaudhry R. Molecular characterization and subtyping of *Blastocystis* sp ecies in irritable bowel syndrome patients from north india. *PLoS ONE.* (2016) 11:e0147055. doi: 10.1371/journal.pone.0147055
35. Khademvatan S, Masjedizadeh R, Rahim F, Mahbodfar H, Salehi R, Yousefi-Razin E, et al. *Blastocystis* and irritable bowel syndrome: frequency and subtypes from Iranian patients. *Parasitol Int.* (2017) 66:142–5. doi: 10.1016/j.parint.2017.01.005
36. Krogsgaard LR, Engsbro AL, Stensvold CR, Nielsen HV, Bytzer P. The prevalence of intestinal parasites is not greater among individuals with irritable bowel syndrome: a population-based case-control study. *Clin Gastroenterol Hepatol.* (2015) 13:507–13.e502. doi: 10.1016/j.cgh.2014.07.065
37. Salvador F, Lobo B, Goterris L, Alonso-Cotoner C, Santos J, Sulleiro E, et al. *Blastocystis* sp. carriage and irritable bowel syndrome: is the association already established? *Biology.* (2021) 10:340. doi: 10.3390/biology10040340
38. Fouad SA, Basyoni MM, Fahmy RA, Kobaisi MH. The pathogenic role of different *Blastocystis hominis* genotypes isolated from patients with irritable bowel syndrome. *Arab J Gastroenterol.* (2011) 12:194–200. doi: 10.1016/j.ajg.2011.11.005
39. Unalan-Altintop T, Vahabov C, Ergunay K, Kurt O, Kav T, Akyon Y, et al. Investigation of *Dientamoeba fragilis* and *Blastocystis* in patients from Turkey with ulcerative colitis and irritable bowel syndrome: any relation with genotypes?. *Acta Trop.* (2022) 231:106451. doi: 10.1016/j.actatropica.2022.106451
40. Peña S, Carrasco G, Rojas P, Castillo D, Ozaki LS, Mercado R. Determination of subtypes of *Blastocystis* sp. in Chilean patients with and without inflammatory bowel syndrome, a preliminary report. *Parasit Epidemiol Cont.* (2020) 8:e00125. doi: 10.1016/j.parepi.2019.e00125
41. Dogruman-Al F, Kustimur S, Yoshikawa H, Tuncer C, Simsek Z, Tanyuksel M, et al. *Blastocystis* subtypes in irritable bowel syndrome and inflammatory bowel disease in Ankara, Turkey. *Mem Instit Oswaldo Cruz.* (2009) 104:724–7. doi: 10.1590/S0074-02762009000500011
42. Jimenez-Gonzalez DE, Martinez-Flores WA, Reyes-Gordillo J, Ramirez-Miranda ME, Arroyo-Escalante S, Romero-Valdovinos M, et al. *Blastocystis* infection is associated with irritable bowel syndrome in a Mexican patient population. *Parasitol Res.* (2012) 110:1269–75. doi: 10.1007/s00436-011-2626-7
43. Vargas-Sanchez GB, Romero-Valdovinos M, Ramirez-Guerrero C, Vargas-Hernandez I, Ramirez-Miranda ME, Martinez-Ocaña J, et al. *Blastocystis* isolates from patients with irritable bowel syndrome and from asymptomatic carriers exhibit similar parasitological loads, but significantly different generation times and genetic variability across multiple subtypes. *PLoS ONE.* (2015) 10:e0124006. doi: 10.1371/journal.pone.0124006
44. Nagel R, Traub RJ, Kwan MM, Bielefeldt-Ohmann H. *Blastocystis* sp ecific serum immunoglobulin in patients with irritable bowel syndrome (IBS) versus healthy controls. *Parasite Vector.* (2015) 8:453. doi: 10.1186/s13071-015-1069-x
45. Olivo-Diaz A, Romero-Valdovinos M, Gudiño-Ramirez A, Reyes-Gordillo J, Jimenez-Gonzalez DE, Ramirez-Miranda ME, et al. Findings related to IL-8 and IL-10 gene polymorphisms in a Mexican patient population with irritable bowel syndrome infected with *Blastocystis*. *Parasitol Res.* (2012) 111:487–91. doi: 10.1007/s00436-012-2830-0
46. Romero-Valdovinos M, Gudiño-Ramírez A, Reyes-Gordillo J, Martínez-Flores WA, Ramírez-Miranda ME, Maravilla P, et al. Interleukin-8 and−10 gene polymorphisms in irritable bowel syndrome. *Mol Biol Rep.* (2012) 39:8837–43. doi: 10.1007/s11033-012-1745-2
47. Xiao QY, Fang XC, Li XQ, Fei GJ. Ethnic differences in genetic polymorphism associated with irritable bowel syndrome. *World J Gastroenterol.* (2020) 26:2049–63. doi: 10.3748/wjg.v26.i17.2049
48. Ismail MH, Molan AL, Abbas SK. Serological levels of cytokines in irritable bowel syndrome (IBS) patients and non-IBS subjects with and without *Blastocystis* spp. infection. *Annal Parasitol.* (2022) 68:77–85. doi: 10.17420/ap6801.411
49. Vara EJ, Brokstad KA, Hausken T, Lied GA. Altered levels of cytokines in patients with irritable bowel syndrome are not correlated with fatigue. *Int J General Med.* (2018) 11:285–91. doi: 10.2147/IJGM.S166600
50. Karamati SA, Mirjalali H, Niyyati M, Yadegar A, Asadzadeh Aghdaei H, Haghighi A, et al. Association of *Blastocystis* ST6 with higher protease activity among symptomatic subjects. *BMC Microbiol.* (2021) 21:285. doi: 10.1186/s12866-021-02341-9
51. Mirza H, Tan KS. *Blastocystis* exhibits inter- and intra-subtype variation in cysteine protease activity. *Parasitol Res.* (2009) 104:355–61. doi: 10.1007/s00436-008-1203-1

52. Mirza H, Wu Z, Teo JD, Tan KS. Statin pleiotropy prevents rho kinase-mediated intestinal epithelial barrier compromise induced by *Blastocystis* cysteine proteases. *Cell Microbiol.* (2012) 14:1474–84. doi: 10.1111/j.1462-5822.2012.01814.x
53. Puthia MK, Lu J, Tan KS. *Blastocystis ratti* contains cysteine proteases that mediate interleukin-8 response from human intestinal epithelial cells in an NF-κB-dependent manner. *Eukaryot Cell.* (2008) 7:435–43. doi: 10.1128/EC.00371-07
54. Mohammad Rahimi H, Yadegar A, Asadzadeh Aghdaei H, Mirjalali H, Zali MR. Modulation of microRNAs and claudin-7 in Caco-2 cell line treated with *Blastocystis* sp., subtype 3 soluble total antigen. *BMC Microbiol.* (2022) 22:111. doi: 10.1186/s12866-022-02528-8
55. Kozieł MJ, Ziaja M, Piastowska-Ciesielska AW. Intestinal barrier, claudins and mycotoxins. *Toxins.* (2021) 13:758. doi: 10.3390/toxins13110758
56. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. *Nature.* (2007) 449:804–10. doi: 10.1038/nature06244
57. Zhu B, Wang X, Li L. Human gut microbiome: the second genome of human body. *Protein Cell.* (2010) 1:718–25. doi: 10.1007/s13238-010-0093-z
58. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, et al. Enterotypes of the human gut microbiome. *Nature.* (2011) 473:174–80. doi: 10.1038/nature09944
59. Goodrich JK, Di Rienzi SC, Poole AC, Koren O, Walters WA, Caporaso JG, et al. Conducting a microbiome study. *Cell.* (2014) 158:250–62. doi: 10.1016/j.cell.2014.06.037
60. Siezen RJ, Kleerebezem M. The human gut microbiome: are we our enterotypes? *Microb Biotechnol.* (2011) 4:550–3. doi: 10.1111/j.1751-7915.2011.00290.x
61. Berbisá MÁ F, Nielsen KR, Ingham AC, Midjord J, Hammer T, et al. Similar gut bacterial composition between patients with ulcerative colitis and healthy controls in a high incidence population: a cross-sectional study of the faroe islands IBD cohort. *Inflamm Bowel Dis*. (2022). doi: 10.1093/ibd/izab355. [Epub ahead of print].
62. McCarthy S, Barrett M, Kirthi S, Pellanda P, Vlckova K, Tobin AM, et al. Altered skin and gut microbiome in hidradenitis suppurativa. *J Invest Dermatol.* (2022) 142:459–68.e415. doi: 10.1016/j.jid.2021.05.036
63. Thompson MD, Kang J, Faerber A, Hinrichs H, Özler O, Cowen J, et al. Maternal obesogenic diet regulates offspring bile acid homeostasis and hepatic lipid metabolism via the gut microbiome in mice. *Am J Physiol Gastrointestinal Liver Physiol.* (2022) 322:G295–309. doi: 10.1152/ajpgi.00247.2021
64. Wang L, Xian YF, Loo SKF, Ip SP, Yang W, Chan WY, et al. Baicalin ameliorates 2,4-dinitrochlorobenzene-induced atopic dermatitis-like skin lesions in mice through modulating skin barrier function, gut microbiota and JAK/STAT pathway. *Bioorgan Chem.* (2022) 119:105538. doi: 10.1016/j.bioorg.2021.105538
65. Janeiro MH, Ramírez MJ, Solas M. Dysbiosis and alzheimer's disease: cause or treatment opportunity? *Cell Mol Neurobiol.* (2022) 42:377–87. doi: 10.1007/s10571-020-01024-9
66. Sheykhsaran E, Abbasi A, Ebrahimzadeh Leylabadlo H, Sadeghi J, Mehri S, Naeimi Mazraeh F, et al. Gut microbiota and obesity: an overview of microbiota to microbial-based therapies. *Postgrad Med J.* (2022). doi: 10.1136/postgradmedj-2021-141311. [Epub ahead of print].
67. Passos M, Moraes-Filho JP. Intestinal microbiota in digestive diseases. *Arq Gastroenterol.* (2017) 54:255–62. doi: 10.1590/s0004-2803.201700000-31
68. Thibaut MM, Bindels LB. Crosstalk between bile acid-activated receptors and microbiome in entero-hepatic inflammation. *Trend Mol Med.* (2022) 28:223–36. doi: 10.1016/j.molmed.2021.12.006
69. Rajilić-Stojanović M, Biagi E, Heilig HG, Kajander K, Kekkonen RA, Tims S, et al. Global and deep molecular analysis of microbiota signatures in fecal samples from patients with irritable bowel syndrome. *Gastroenterology.* (2011) 141:1792–801. doi: 10.1053/j.gastro.2011.07.043
70. Shankar V, Reo NV, Paliy O. Simultaneous fecal microbial and metabolite profiling enables accurate classification of pediatric irritable bowel syndrome. *Microbiome.* (2015) 3:73. doi: 10.1186/s40168-015-0139-9
71. Tap J, Derrien M, Törnblom H, Brazeilles R, Cools-Portier S, Doré J, et al. Identification of an intestinal microbiota signature associated with severity of irritable bowel syndrome. *Gastroenterology.* (2017) 152:111–23.e118. doi: 10.1053/j.gastro.2016.09.049
72. Bhattarai Y, Muniz Pedrogo DA, Kashyap PC. Irritable bowel syndrome: a gut microbiota-related disorder? *Am J Physiol Gastrointestinal Liver Physiol.* (2017) 312:G52–62. doi: 10.1152/ajpgi.00338.2016
73. Zhuang X, Tian Z, Li L, Zeng Z, Chen M, Xiong L. Fecal microbiota alterations associated with diarrhea-predominant irritable bowel syndrome. *Front Microbiol.* (2018) 9:1600. doi: 10.3389/fmicb.2018.01600
74. Nagel R, Traub RJ, Allcock RJ, Kwan MM, Bielefeldt-Ohmann H. Comparison of faecal microbiota in *Blastocystis*-positive and *Blastocystis*-negative irritable bowel syndrome patients. *Microbiome.* (2016) 4:47. doi: 10.1186/s40168-016-0191-0
75. Shukla R, Ghoshal U, Dhole TN, Ghoshal UC. Fecal microbiota in patients with irritable bowel syndrome compared with healthy controls using real-time polymerase chain reaction: an evidence of dysbiosis. *Dig Dis Sci.* (2015) 60:2953–62. doi: 10.1007/s10620-015-3607-y
76. Mei L, Zhou J, Su Y, Mao K, Wu J, Zhu C, et al. Gut microbiota composition and functional prediction in diarrhea-predominant irritable bowel syndrome. *BMC Gastroenterol.* (2021) 21:105. doi: 10.1186/s12876-021-01693-w
77. Pittayanon R, Lau JT, Yuan Y, Leontiadis GI, Tse F, Surette M, et al. Gut microbiota in patients with irritable bowel syndrome-a systematic review. *Gastroenterology.* (2019) 157:97–108. doi: 10.1053/j.gastro.2019.03.049
78. Lo Presti A, Zorzi F, Del Chierico F, Altomare A, Cocca S, Avola A, et al. Fecal and mucosal microbiota profiling in irritable bowel syndrome and inflammatory bowel disease. *Front Microbiol.* (2019) 10:1655. doi: 10.3389/fmicb.2019.01655
79. Zhu X, Hong G, Li Y, Yang P, Cheng M, Zhang L, et al. Understanding of the site-specific microbial patterns towards accurate identification for patients with diarrhea-predominant irritable bowel syndrome. *Microbiol Spect.* (2021) 9:e0125521. doi: 10.1128/Spectrum.01255-21
80. Sciavilla P, Strati F, Di Paola M, Modesto M, Vitali F, Cavalieri D, et al. Gut microbiota profiles and characterization of cultivable fungal isolates in IBS patients. *Appl Microbiol Biotechnol.* (2021) 105:3277–88. doi: 10.1007/s00253-021-11264-4
81. Lopetuso LR, Petito V, Graziani C, Schiavoni E, Paroni Sterbini F, Poscia A, et al. Gut microbiota in health, diverticular disease, irritable bowel syndrome, and inflammatory bowel diseases: time for microbial marker of gastrointestinal disorders. *Dig Dis.* (2018) 36:56–65. doi: 10.1159/000477205
82. Sundin J, Rangel I, Fuentes S, Heikamp-de Jong I, Hultgren-Hörnquist E, de Vos WM, et al. Altered faecal and mucosal microbial composition in post-infectious irritable bowel syndrome patients correlates with mucosal lymphocyte phenotypes and psychological distress. *Alim Pharmacol Therap.* (2015) 41:342–51. doi: 10.1111/apt.13055
83. Giamarellos-Bourboulis E, Tang J, Pyleris E, Pistiki A, Barbatzas C, Brown J, et al. Molecular assessment of differences in the duodenal microbiome in subjects with irritable bowel syndrome. *Scandinavian J Gastroenterol.* (2015) 50:1076–87. doi: 10.3109/00365521.2015.1027261
84. Liu Y, Zhang L, Wang X, Wang Z, Zhang J, Jiang R, et al. Similar fecal microbiota signatures in patients with diarrhea-predominant irritable bowel syndrome and patients with depression. *Clin Gastroenterol Hepatol.* (2016) 14:1602–11.e1605. doi: 10.1016/j.cgh.2016.05.033
85. Carroll IM, Ringel-Kulka T, Keku TO, Chang YH, Packey CD, Sartor RB, et al. Molecular analysis of the luminal- and mucosal-associated intestinal microbiota in diarrhea-predominant irritable bowel syndrome. *Am J Physiol Gastrointestinal Liver Physiol.* (2011) 301:G799–807. doi: 10.1152/ajpgi.00154.2011
86. Drossman DA. Functional gastrointestinal disorders: history, pathophysiology, clinical features and rome IV. *Gastroenterology.* (2016) 150:1262–1279.e2. doi: 10.1053/j.gastro.2016.02.032
87. Muller PA, Koscsó B, Rajani GM, Stevanovic K, Berres ML, Hashimoto D, et al. Crosstalk between muscularis macrophages and enteric neurons regulates gastrointestinal motility. *Cell.* (2014) 158:300–13. doi: 10.1016/j.cell.2014.04.050
88. Barbara G, Stanghellini V, De Giorgio R, Cremon C, Cottrell GS, Santini D, et al. Activated mast cells in proximity to colonic nerves correlate

with abdominal pain in irritable bowel syndrome. *Gastroenterology.* (2004) 126:693–702. doi: 10.1053/j.gastro.2003.11.055
89. Ohman L, Isaksson S, Lindmark AC, Posserud I, Stotzer PO, Strid H, et al. T-cell activation in patients with irritable bowel syndrome. *Am J Gastroenterol.* (2009) 104:1205–12. doi: 10.1038/ajg.2009.116
90. Nasser Y, Petes C, Simmers C, Basso L, Altier C, Gee K, et al. Activation of peripheral blood CD4+ T-cells in ibs is not associated with gastrointestinal or psychological symptoms. *Sci Rep.* (2019) 9:3710. doi: 10.1038/s41598-019-40124-5
91. Vicario M, González-Castro AM, Martínez C, Lobo B, Pigrau M, Guilarte M, et al. Increased humoral immunity in the jejunum of diarrhoea-predominant irritable bowel syndrome associated with clinical manifestations. *Gut.* (2015) 64:1379–88. doi: 10.1136/gutjnl-2013-306236
92. Chatoo M, Li Y, Ma Z, Coote J, Du J, Chen X. Involvement of corticotropin-releasing factor and receptors in immune cells in irritable bowel syndrome. *Front Endocrinol.* (2018) 9:21. doi: 10.3389/fendo.2018.00021
93. Lobo B, Ramos L, Martínez C, Guilarte M, González-Castro AM, Alonso-Cotoner C, et al. Downregulation of mucosal mast cell activation immune response in diarrhoea-irritable bowel syndrome by oral disodium cromoglycate: a pilot study. *United Europ Gastroenterol J.* (2017) 5:887–97. doi: 10.1177/2050640617691690
94. Medzhitov R. Recognition of microorganisms and activation of the immune response. *Nature.* (2007) 449:819–26. doi: 10.1038/nature06246
95. Bertani B, Ruiz N. Function and biogenesis of lipopolysaccharides. *EcoSal Plus.* (2018) 8:10.1128/ecosalplus.ESP-0001-2018. doi: 10.1128/ecosalplus.ESP-0001-2018
96. Park BS, Lee JO. Recognition of lipopolysaccharide pattern by TLR4 complexes. *Exp Mol Med.* (2013) 45:e66. doi: 10.1038/emm.2013.97
97. Anitha M, Vijay-Kumar M, Sitaraman SV, Gewirtz AT, Srinivasan S. Gut microbial products regulate murine gastrointestinal motility via Toll-like receptor 4 signaling. *Gastroenterology.* (2012) 143:1006–16.e1004. doi: 10.1053/j.gastro.2012.06.034
98. Brint EK, MacSharry J, Fanning A, Shanahan F, Quigley EM. Differential expression of toll-like receptors in patients with irritable bowel syndrome. *Am J Gastroenterol.* (2011) 106:329–36. doi: 10.1038/ajg.2010.438
99. Belmonte L, Beutheu Youmba S, Bertiaux-Vandaële N, Antonietti M, Lecleire S, Zalar A, et al. Role of toll like receptors in irritable bowel syndrome: differential mucosal immune activation according to the disease subtype. *PLoS ONE.* (2012) 7:e42777. doi: 10.1371/journal.pone.0042777
100. Jalanka J, Lam C, Bennett A, Hartikainen A, Crispie F, Finnegan LA, et al. Colonic gene expression and fecal microbiota in diarrhea-predominant irritable bowel syndrome: increased toll-like receptor 4 but minimal inflammation and no response to mesalazine. *J Neurogastroenterol Motil.* (2021) 27:279–91. doi: 10.5056/jnm20205
101. Yañez CM, Hernández AM, Sandoval AM, Domínguez MAM, Muñiz SAZ, Gómez JOG. Prevalence of *Blastocystis* and its association with Firmicutes/Bacteroidetes ratio in clinically healthy and metabolically ill subjects. *BMC Microbiol.* (2021) 21:339. doi: 10.1186/s12866-021-02402-z
102. Kim MJ, Lee YJ, Kim TJ, Won EJ. Gut microbiome profiles in colonizations with the enteric protozoa *Blastocystis* in Korean populations. *Microorganisms.* (2021) 10:34. doi: 10.3390/microorganisms10010034
103. Deng L, Tan K. Interactions between *Blastocystis* subtype ST4 and gut microbiota *in vitro*. *Parasit Vector.* (2022) 15:80. doi: 10.1186/s13071-022-05194-x
104. Deng L, Wojciech L, Png CW, Koh EY, Aung TT, Kioh D, et al. Experimental colonization with *Blastocystis* ST4 is associated with protective immune responses modulation of gut microbiome in a DSS-induced colitis mouse model. *Cell Mol Life Sci.* (2022) 79:245. doi: 10.1007/s00018-022-04271-9
105. Stensvold CR, Sørland BA, Berg R, Andersen LO, van der Giezen M, Bowtell JL, et al. Stool microbiota diversity analysis of *Blastocystis*-positive and *Blastocystis*-negative individuals. *Microorganisms.* (2022) 10:326. doi: 10.3390/microorganisms10020326
106. Nourrisson C, Scanzi J, Pereira B, NkoudMongo C, Wawrzyniak I, Cian A, et al. *Blastocystis* is associated with decrease of fecal microbiota protective bacteria: comparative analysis between patients with irritable bowel syndrome and control subjects. *PLoS ONE.* (2014) 9:e111868. doi: 10.1371/journal.pone.0111868
107. Defaye M, Nourrisson C, Baudu E, Lashermes A, Meynier M, Meleine M, et al. Fecal dysbiosis associated with colonic hypersensitivity and behavioral alterations in chronically *Blastocystis*-infected rats. *Sci Rep.* (2020) 10:9146. doi: 10.1038/s41598-020-66156-w
108. Nourrisson C, Scanzi J, Brunet J, Delbac F, Dapoigny M, Poirier P. Prokaryotic and eukaryotic fecal microbiota in irritable bowel syndrome patients and healthy individuals colonized with *Blastocystis*. *Front Microbiol.* (2021) 12:713347. doi: 10.3389/fmicb.2021.713347
109. Beheshti-Maal A, Shahrokh S, Ansari S, Mirsamadi ES, Yadegar A, Mirjalali H, et al. Gut mycobiome: the probable determinative role of fungi in IBD patients. *Mycoses.* (2021) 64:468–76. doi: 10.1111/myc.13238
110. Das A, O'Herlihy E, Shanahan F, O'Toole PW, Jeffery IB. The fecal mycobiome in patients with irritable bowel syndrome. *Sci Rep.* (2021) 11:124. doi: 10.1038/s41598-020-79478-6
111. Gu Y, Zhou G, Qin X, Huang S, Wang B, Cao H. The potential role of gut mycobiome in irritable bowel syndrome. *Front Microbiol.* (2019) 10:1894. doi: 10.3389/fmicb.2019.01894
112. Deng L, Wojciech L, Gascoigne N, Peng G, Tan K. New insights into the interactions between *Blastocystis*, the gut microbiota, and host immunity. *PLoS Pathog.* (2021) 17:e1009253. doi: 10.1371/journal.ppat.1009253
113. Jeffery IB, Cotter PD, Scanlan PD. Collateral damage in the human gut microbiome - *Blastocystis* is significantly less prevalent in an antibiotic-treated adult population compared to non-antibiotic treated controls. *Front Cell Infect Microbiol.* (2022) 12:822475. doi: 10.3389/fcimb.2022.822475

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers | Frontiers in Medicine

TYPE Brief Research Report
PUBLISHED 01 August 2022
DOI 10.3389/fmed.2022.964640

OPEN ACCESS

EDITED BY
Milad Badri,
Qazvin University of Medical Sciences, Iran

REVIEWED BY
Morteza Saki,
Ahvaz Jundishapur University of Medical Sciences, Iran
F.-X. Campbell-Valois,
University of Ottawa, Canada

*CORRESPONDENCE
Karrie K. K. Ko
karrie_ko@gis.a-star.edu.sg;
karrie.ko.k.k@singhealth.com

SPECIALTY SECTION
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

RECEIVED 08 June 2022
ACCEPTED 08 July 2022
PUBLISHED 01 August 2022

CITATION
Ko KKK, Chu JJK, Lim KM, Yingtaweesittikul H, Huang W, Tan SYL, Goh KCM, Tan SH, Ng TY, Maiwald M, Chia JWZ, Cao DY, Tan YE, Sim JHC, Koh TH, Nagarajan N and Suphavilai C (2022) Clonal serotype 1c multidrug-resistant *Shigella flexneri* detected in multiple institutions by sentinel-site sequencing. *Front. Med.* 9:964640. doi: 10.3389/fmed.2022.964640

Clonal serotype 1c multidrug-resistant *Shigella flexneri* detected in multiple institutions by sentinel-site sequencing

Karrie K. K. Ko[1,2,3,4,5]*, Joash Jun Keat Chu[3], Kar Mun Lim[3], Hatairat Yingtaweesittikul[6], Wenjie Huang[1,5], Shireen Yan Ling Tan[1,5], Kenneth Choon Meng Goh[1,5], Si Huei Tan[7], Tong Yong Ng[8], Matthias Maiwald[4,5,9], Jonathan Wei Zhong Chia[10], Delphine Yanhong Cao[1], Yen Ee Tan[1,5], James Heng Chiak Sim[1,5], Tse Hsien Koh[1,5], Niranjan Nagarajan[3] and Chayaporn Suphavilai[3]

[1]Department of Microbiology, Singapore General Hospital, Singapore, Singapore, [2]Department of Molecular Pathology, Singapore General Hospital, Singapore, Singapore, [3]Genome Institute of Singapore, Agency for Science, Technology and Research, Singapore, Singapore, [4]Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore, [5]Duke-NUS Medical School, Singapore, Singapore, [6]Advanced Research Center for Computational Simulation, Chiang Mai University, Chiang Mai, Thailand, [7]Department of Laboratory Medicine, Changi General Hospital, Singapore, Singapore, [8]Department of Pathology, Sengkang General Hospital, Singapore, Singapore, [9]Department of Pathology and Laboratory Medicine, KK Women's and Children's Hospital, Singapore, Singapore, [10]Department of Laboratory Medicine, Tan Tock Seng Hospital, Singapore, Singapore

Shigella flexneri is a major diarrhoeal pathogen, and the emergence of multidrug-resistant *S. flexneri* is of public health concern. We report the detection of a clonal cluster of multidrug-resistant serotype 1c (7a) *S. flexneri* in Singapore in April 2022. Long-read whole-genome sequence analysis found five *S. flexneri* isolates to be clonal and harboring the extended-spectrum β-lactamases $bla_{CTX-M-15}$ and bla_{TEM-1}. The isolates were phenotypically resistant to ceftriaxone and had intermediate susceptibility to ciprofloxacin. The *S. flexneri* clonal cluster was first detected in a tertiary hospital diagnostic laboratory (sentinel-site), to which the *S. flexneri* isolates were sent from other hospitals for routine serogrouping. Long-read whole-genome sequence analysis was performed in the sentinel-site near real-time in view of the unusually high number of *S. flexneri* isolates received within a short time frame. This study demonstrates that near real-time sentinel-site sequence-based surveillance of convenience samples can detect possible clonal outbreak clusters and may provide alerts useful for public health mitigations at the earliest possible opportunity.

KEYWORDS

***Shigella flexneri*, multidrug-resistant (MDR), surveillance, whole-genome sequence (WGS), outbreak**

Introduction

Shigella is a major diarrhoeal pathogen and the second leading cause of diarrhoeal mortality globally (1). The pathogen is transmitted through the fecal-oral route, and adults can be infected by as few as 10 bacilli (2), making it highly transmissible. The clinical manifestations of *Shigella* infection range from self-limited diarrhea to fulminant dysentery and life-threatening invasive systemic infections. Shigellosis is estimated to cause more than 200,000 deaths annually, and the greatest burden of morbidity and mortality is among children below the age of five in lower-middle-income countries, as well as adults aged 70 and older (1, 3, 4).

Among the four *Shigella* subgroups (*Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii,* and *Shigella sonnei*), *S. flexneri* and *S. sonnei* cause the majority of laboratory-confirmed *Shigella* infections in developing countries and developed countries, respectively (5, 6). *Shigella flexneri* thus incurs a substantial disease burden among disadvantaged populations worldwide. Further complicating the control of *S. flexneri* infections is the increasing resistance to third-generation cephalosporins in Asia (7–11), and the emergence and dissemination of multidrug-resistant (MDR) *S. flexneri* strains, particularly among men who have sex with men (MSM) (12).

The rise of resistance against third-generation cephalosporins, ciprofloxacin, and azithromycin, coupled with the highly transmissible nature of *S. flexneri*, therefore represents a significant public health threat. Fast and accurate detection of *S. flexneri* clusters enables rapid investigation of outbreak sources and early mitigating actions, thereby enabling public health actions at the earliest opportunity.

Traditional diarrhoeal illness surveillance relies on syndrome-based as well as laboratory-based notification of public health authorities. However, jurisdictions differ in terms of notification requirements for *Shigella* infections. For instance, the UK Health Security Agency requires healthcare practitioners to notify the relevant public health agencies when a case of *Shigella* infection is detected (13), whereas some other jurisdictions do not (14). Where notification is not legally required, the public health system relies on astute individual healthcare practitioners to make the decision to notify the public health agency when a *Shigella* cluster is suspected. Such reliance on individual decisions in a complex healthcare system carries disadvantages. Most clinicians will only be aware of cases within their area of work and unable to identify disease trends, and this contributes to gaps in disease reporting and trend analyses.

Early detection of potential point-source outbreaks is of particular importance, as the detection and removal of the offending source is essential for control (15, 16). Traditional investigation of the intra-species relatedness of diarrhoeal pathogens such as *Shigella* relies on various typing procedures performed in reference public health laboratories. In jurisdictions where *Shigella* isolates are not routinely transferred to a reference laboratory, isolate viability may be compromised, leading to a loss of time and data that are essential for outbreak investigation.

To address this public health gap, we implemented a decentralized, sentinel-site whole-genome sequencing (WGS) capability within a routine diagnostic laboratory in a tertiary hospital (Hospital IV). Hospital IV routinely receives *Shigella* isolates from several hospitals for identity confirmation and/or serogrouping, and is a site that can perform sentinel surveillance. To contain cost and shorten the turnaround time, the Nanopore MinION sequencer (Oxford Nanopore Technologies, United Kingdom) was chosen for sentinel-site sequencing. We hypothesize that the sentinel-site sequence-based surveillance will reduce the time taken to detect a clonal outbreak of *S. flexneri* and that this strategy may provide for early alerts for public health mitigations.

Methods

Microbiological investigations

Stool culture isolates identified in Hospital I and Hospital II to be *Shigella* species or *Shigella flexneri* were transferred to Hospital IV for identity confirmation and/or serogrouping. Identity was confirmed in Hospital IV using Vitek 2 GN cards (bioMérieux, Marcy-l'Étoile, France), and observation of motility (–), indole production (+/–), and lysine decarboxylase activity (–) (17). *Shigella* serogrouping was performed using a set of commercial *Shigella* antisera (Mast® Assure antiserum Shigella, Mast Group, Bootle, UK). Antimicrobial susceptibility testing was performed using a combination of disk diffusion, Etest (bioMerieux, Marcy-l'Étoile, France) and Vitek® 2 susceptibility testing (bioMerieux, Marcy-l'Étoile, France). Antimicrobial susceptibility tests were performed and interpreted in accordance with the Clinical and Laboratory Standards Institute (CLSI) standards in Hospitals I, II, IV, and V (18). Hospital III used both CDS (19) and CLSI (18) standards for susceptibility testing. Detailed description of the list of antibiotics tested are summarized in Supplementary Table 1. All isolates identified to be *Shigella flexneri* from 10th March 2022 to 13th April 2022 in Hospitals I-V were included in this study.

Whole-genome sequencing

Three *S. flexneri* isolates from Hospital I, two *S. flexneri* isolates from Hospital II, and one control *Escherichia coli* isolate (ATCC25922) were subjected to DNA extraction using DNeasy Powersoil Pro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA samples were prepared using the Q20+ Native Barcoding Kit (SQK-NDB112.24), according to the "Ligation sequencing gDNA -

native barcoding (SQKNBD112.24)" protocol. Four hundred nanogram of genomic DNA per sample was used directly for DNA repair and end preparation, without any prior shearing. The samples were incubated at 20 and 65°C for 15 and 5 min, respectively, during the DNA repair and end preparation. The incubation periods for native barcode ligation and adaptor ligation were extended to 30 min. Throughout the protocol, samples were mixed by gentle flicking of the microcentrifuge tube, instead of pipetting. AMPure XP beads (Beckman Coulter, USA) incubation on the revolver rotator (Labnet, USA) was extended to 10 min, and samples were eluted at 37°C for 10 min. To maximize the yield of the libraries obtained, 200 ng of pooled samples were used for adaptor ligation. Each prepared library was loaded into a R10.4 MinION flow cell (FLO-MIN112) and sequenced on a MinION Mk1c machine for >16 h. The acquisition of the sequenced reads was carried out using MinKNOW v21.11.6. For each sample, at least 100× coverage was obtained and used for downstream analysis. Basecalling, demultiplexing and trimming of barcodes and adaptor sequences were carried out *via* the Guppy v6.1.1 Super High Accuracy basecaller (Oxford Nanopore Technologies).

Sequence data analysis

The trimmed, filtered reads with an average Phred score >10 were assembled with Flye v2.9 (20) and polished twice with medaka v1.6 (Oxford Nanopore Technologies). The resultant draft genomes were used for downstream analysis. The genome data of the five *S. flexneri* isolates were submitted to the GenBank database under the BioProject ID PRJNA841078, with BioSample accessions SAMN28576597, SAMN28576598, SAMN28576599, SAMN28576600, and SAMN28576601.

Parsnp v1.2 (21) was used for phylogenetic analysis. Fifty-three complete *S. flexneri* reference genomes were downloaded from NCBI (22). All genomes with genetic markers for lacY and without ipaH were removed. The remaining 16 complete reference genomes and five query genomes were included in the phylogenetic analysis. Core genome alignment and single-nucleotide polymorphism (SNP) calling for all included genomes were performed against the reference genome NZ_LR213452.1, with Parsnp's PhiPack (23) option (parsnp-x) to identify regions of recombination. A maximum-likelihood phylogenetic tree was constructed with core genome SNPs (RAxML-NG v1.1.0 with 500 bootstrap replicates). Whole-genome average nucleotide identity (ANI) values were calculated using MUMmer 3.0 (24) alignments.

To search for highly similar *S. flexneri* whole-genome sequences in accessible databases, 9,255 *S. flexneri* whole-genome sequences were downloaded from NCBI (accessed on 17th April 2022) (22). MUMmer 3.0 (24) was used for pairwise alignment between the five query genomes and the 9,255 downloaded *S. flexneri* whole-genome sequences. The top 200 genomes with the highest ANI values, averaged across five query genomes, were then included in the subsequent phylogenetic analysis using Parsnp v1.2 (RAxML-NG v1.1.0 with 500 bootstrap replicates).

ShigaTyper (25) was used for *in silico* serotyping with the latest database (11 February 2022). *In silico* multilocus sequence types (MLST) were determined using publicly available tools in Pathogenwatch (https://pathogen.watch/) using the Enterobase Escherichia/Shigella MLST scheme (https://enterobase.readthedocs.io/en/latest/mlst/mlst-legacy-info-ecoli.html). Genetic markers for antimicrobial resistance and virulence factors were identified using AMRFinderPlus v3.10.24 (26).

Results

Timeline of *S. flexneri* detection and analysis in five hospitals

Figure 1 summarizes the timeline of the detection of eight known *S. flexneri* cases within a 5-week period. Five *S. flexneri* isolates were independently obtained by Hospital I (Cases A, B, and D) and Hospital II (Cases C and E) from 10th March to 13th April 2022. Hospital IV (sentinel-site) received Cases A, B and C for identity confirmation and/or serogrouping within a 2-day period. Cases A, B and C (* in Figure 1) were sequenced near real-time in hospital IV. Once a clonal cluster was suspected based on initial results, communications within the informal hospital laboratory network established that there were at least five additional *S. flexneri* cases from March 2022 to April 2022 among five hospitals. Cases D and E (^ in Figure 1) were sequenced subsequently. The isolates of cases F, G, and H had been discarded and were not available for further analysis.

In total, five *S. flexneri* isolates (Case A–E) were included in the sentinel-site sequence analysis. Antibiograms were retrieved from the remaining three cases (Cases F, G, and H). While not mandatory, the originating laboratories had made individual voluntary notifications to the public health agency at the time of diagnosis for each case (Figure 2A), but the recognition that there was a case cluster occurred at the tertiary hospital (Hospital IV) laboratory.

Decentralized laboratory-based sentinel surveillance reduced time taken to detect possible clonal outbreak

Current laboratory-based surveillance for pathogens such as *S. flexneri* relies on investigations in central reference laboratories. For diseases and pathogen isolates not routinely notifiable and transferred to the public health reference laboratory, investigations may be delayed due to the time

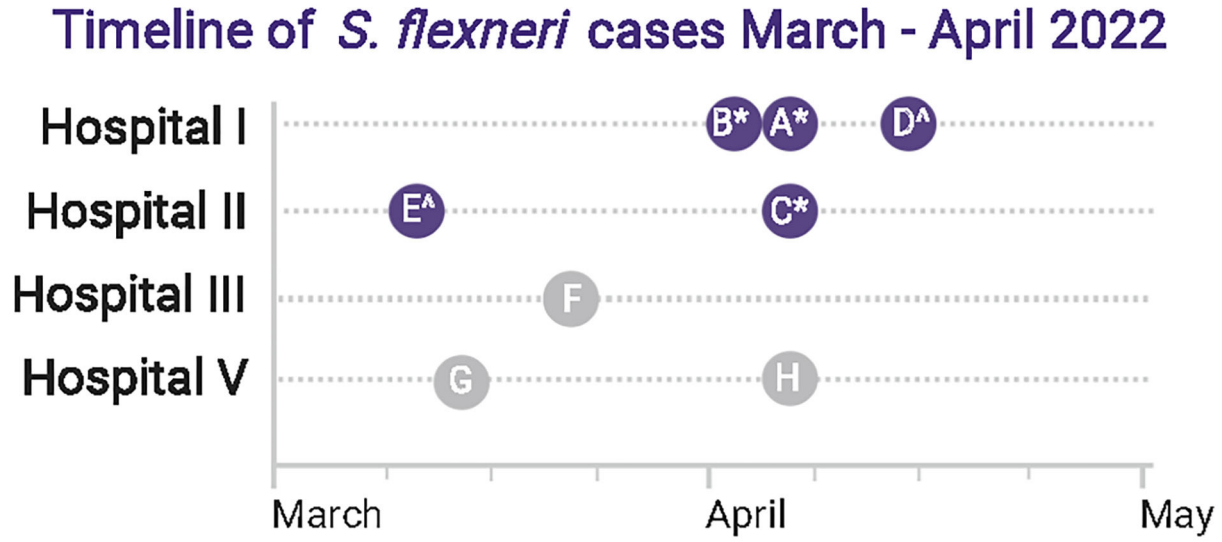

FIGURE 1
Summary of *Shigella flexneri* detection timeline. There were eight known laboratory-proven *S. flexneri* cases (Cases A–H) in four hospitals within a 5-week period. Five isolates were sequenced in this study (Cases A–E, dark blue circle), among which 3 (*) were sequenced near real-time. Two additional isolates were sequenced subsequently (^). Isolates of three cases from Hospitals III and V (Cases F, G, H) had been discarded and were not available for sequence analysis (gray circle). BioRender was used to create this figure.

taken for unusual disease trend detection and sample transfers (Figure 2A). Figure 2B illustrates the sentinel surveillance model we used in the current study. An existing network of hospital diagnostic laboratories routinely transfers *Shigella* spp. isolates to a tertiary hospital diagnostic laboratory for consolidated serogrouping and/or identity confirmation. In this study, the same isolates received for diagnostic testing were subjected to WGS within the same laboratory near real-time. The isolates under investigation were found to be genetically closely related within five working days (Day 5) of receipt. On Day 12, the whole-genome comparison results were formally forwarded to the local public health agency. The Singapore Ministry of Health subsequently issued a notification to hospitals (Day 14) to request for archived *Shigella* isolates and any associated data from 2018 to 2022 to be forwarded to the public health agency.

S. flexneri isolates under investigation were genetically closely related

Phylogenetic analysis of the five *S. flexneri* isolates received from two institutions showed that they formed a distinct cluster (Figure 3A). Sixteen other complete *S. flexneri* genomes were included in the core genome phylogenetic analysis using Parsnp (21). A maximum of 20 core genome SNPs differences were observed among the isolates under investigation. The core SNPs matrix is available in Supplementary Table 2. Among the complete chromosomes included in the phylogenetic analysis, the *S. flexneri* strain AUSMDU00008355 (Genbank accession: LR213452.1) was found to be genetically most closely related to the Singapore isolates in this study. AUSMDU00008355 was 172–181 core genome SNPs relative to the Singaporean isolates, and was isolated in 2016 (was made publicly available in 2019) from the stool sample of a symptomatic individual in Australia (27). Pairwise comparisons of the five Singaporean isolates showed that whole-genome average nucleotide identity (ANI) among the five Singaporean isolates was >99.999%, indicating that they were likely to be clonal in nature (Supplementary Figure 1). The whole-genome similarities and SNP matrices of the five query genomes and 16 reference genomes are summarized in Supplementary Figures 1–3.

In silico typing was performed to obtain the serotypes and multilocus sequence types (MLST) of the query and reference genomes. *In silico* serotype prediction with ShigaTyper (25) found all five isolates to belong to *S. flexneri* serotype 1c (7a) (Figure 3B). Based on the ShigaTyper reference sequence database, all five query isolates contain the genetic markers for *S. flexneri* specific O-antigen flippase (*Sf_wzx*) and polymerase (*Sf_wzy*). The presence of O-antigen modification genetic markers, namely glucosyltransferases *gtrI* and *gtrIC*, and the absence of O-antigen acetylase 1b (*Oac1b*), gave rise to the *in silico* designation of *S. flexneri* serotype 1c (7a) for all five isolates. Consistent with the species identification and invasive nature of these clinical isolates, the genetic markers for invasive plasmid antigen B (*ipaB*) and invasive plasmid antigen H (*ipaH*) were present in all five isolates. Of note, members of the *ipaH* gene family share a conserved C-terminal catalytic domain, and the term "*ipaH*" used in Figure 3B represents matches to

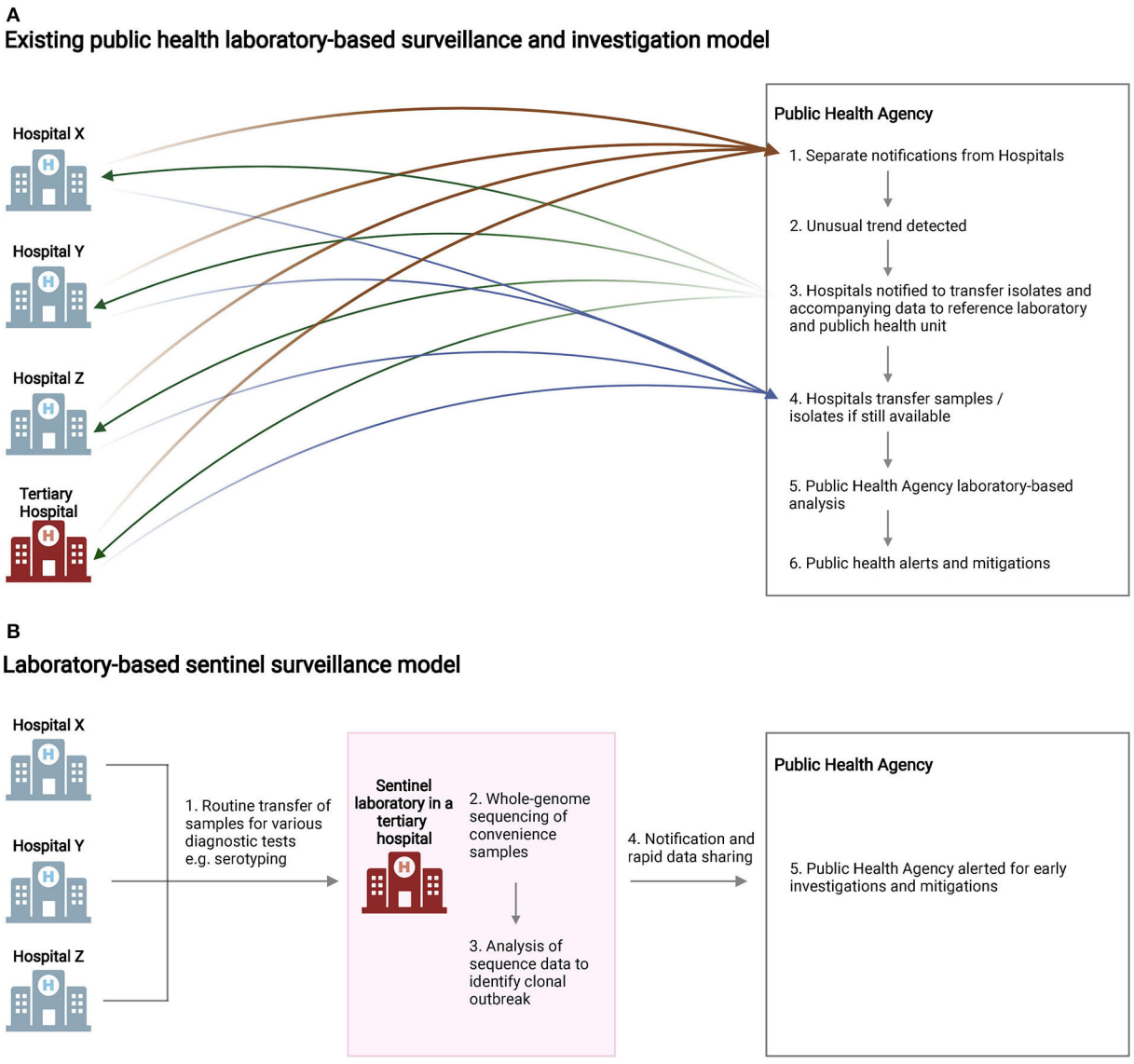

FIGURE 2
Summary of laboratory-based surveillance models. **(A)** For diseases and isolates not routinely notifiable and transferred to central reference laboratories, delays in outbreak detection and laboratory investigations may occur due to logistical challenges. **(B)** Sentinel surveillance of convenience samples, implemented in the current study, enabled the streamlined detection of clonal isolates of public health importance and provided early alerts for the public health agency. BioRender was used to create this figure.

this conserved 780 bp region of *ipaH* (designated as *ipaH_c* in the ShigaTyper database). This conserved region of *ipaH* was detected in the chromosome and the large virulence plasmid of all five genomes under investigation. Ten other complete genomes downloaded from NCBI contained this conserved 780 bp region of *ipaH* in the chromosome and virulence plasmids. The remaining six complete genomes downloaded from NCBI did not contain sufficient information for us to delineate if the conserved 780 bp region of *ipaH* was found in the chromosome or plasmid.

Using the Enterobase *Escherichia/Shigella* MLST scheme, all five isolates were found to belong to the ST245 clonal complex. All five isolates had identical allelic profiles based on gene sequences of seven housekeeping loci (adenylate kinase *adk*, fumarate hydratase *fumC*, DNA gyrase *gyrB*, isocitrate/isopropylmalate dehydrogenase *icd*, malate dehydrogenase *mdh*, adenylosuccinate dehydrogenase *purA*, ATP/GTP binding motif *recA*). The full allelic profiles of all isolates under investigation and reference genomes are summarized in Supplementary Table 3.

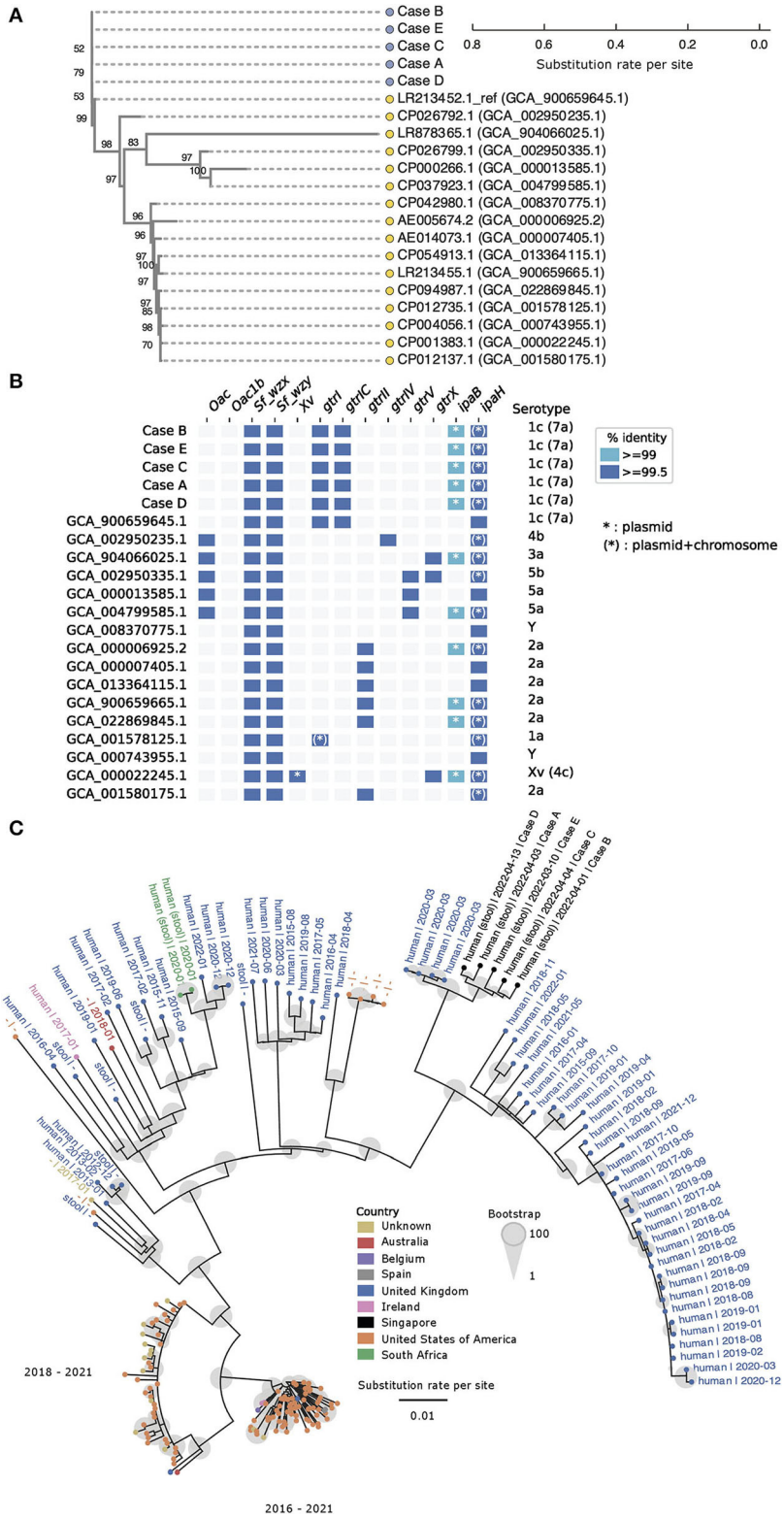

FIGURE 3
Shigella flexneri isolates under investigation are genetically closely related and form a distinct phylogenetic cluster. **(A)** Core genome single nucleotide polymorphisms (SNP) phylogenetic tree generated by Parsnp illustrates phylogenetic relatedness of 16 complete reference genomes

(Continued)

FIGURE 3
(yellow circle) and five query genomes (blue circles). The scale represents substitution rate per site, and the number at each branch indicates the bootstrap support value. **(B)** *in silico* serotyping with ShigaTyper suggests all query isolates are of serotype 1c (7a). The horizontal axis lists ShigaTyper determinants that contributed to the eventual *in silico* serotype (vertical axis). The term "*ipaH*" in this figure represents a conserved 780 bp region of *ipaH* encoding for the C-terminal catalytic domain, which was originally designated as "*ipaH_c*" in the ShigaTyper database. **(C)** Parsnp phylogenetic tree constructed with five Singapore query genomes and 200 most relevant genomes out of 9,255 publicly available *Shigella flexneri* genomes. The scale represents substitution rate per site, and the size of the gray circle at each branch indicates the bootstrap support value. Each genome sequence is represented by a solid circle (●), and the color of the circle represents the country from which the genome sequence was reported. Genomes that are phylogenetically more closely related to the Singaporean isolates (black circles) are labeled with basic metadata in the following format (sample source | date of sample collection in YYYY-MM). For optimal visualization, genomes that were phylogenetically more distant from the Singaporean isolates were represented by colored solid circles without associated metadata. *Oac*, *S. flexneri*-specific *O*-acetyltransferase gene marker; Oac1b, *S. flexneri*-specific O-acetyltransferase gene marker specific for serotype 1b and 7b; *Sf_wzx*, *S. flexneri*-specific O-antigen flippase gene marker; *Sf_wzy*, *S. flexneri*-specific O-antigen polymerase gene marker; *Xv*, *S. flexneri*-specific gene marker encoding for protein homolog of LTA synthase family protein; *gtrI*, glucosyltransferase mediating addition of first glucosyl group to the O-antigen backbone in *S. flexneri* serotypes 1 and 7; *gtrIC*, glucosyltransferase mediating addition of second glucosyl group to the O-antigen backbone in *S. flexneri* serotypes 1c (7a); *grtII*, glucosyltransferase of *S. flexneri* serotype 2; *gtrIV*, glucosyltransferase of *Shigella flexneri* serotype 4; *gtrV*, glucosyltransferase of *S. flexneri* serotype 5; *gtrX*, glucosyltransferase of serotypes 2 or X; *ipaB*, invasive plasmid antigen B; *IpaH*, a 780 bp region of the *ipaH* genes encoding the highly conserved C-terminal catalytic domain.

To search for other highly similar *S. flexneri* genomes, the five Singapore *S. flexneri* genomes were compared to 9,255 downloaded whole-genome sequences. Among these, 200 public whole-genome sequences with the highest ANI to the five query genomes were selected for subsequently phylogenetic analysis. The five Singapore *S. flexneri* isolates formed a distinct cluster and were found to be most closely related (ANI ≥99.999%, Supplementary Table 4) to a cluster of four assemblies submitted by Public Health England (currently known as the UK Health Security Agency; GCA_013457635.1, GCA_013457815.1, GCA_013457355.10 and GCA_013455205.1; Figure 3C, Supplementary Table 4). All four samples were collected from individuals in the United Kingdom in March 2020 and the sequences were submitted as part of the routine surveillance of *Escherichia coli* and *Shigella* (27).

S. flexneri isolates under investigation contain identical genetic markers for antimicrobial resistance (AMR) and virulence factors

Antimicrobial susceptibility testing showed that Cases A–E were multidrug-resistant (MDR) and had identical categorical AMR profiles based on CLSI M100 (32nd edition) (18) interpretation (Figure 4A). All five isolates were resistant to ceftriaxone, with minimum inhibitory concentration (MIC) values ranging from 16 to 32 mg/L. The isolates also demonstrated resistance to cotrimoxazole and intermediate resistance to ciprofloxacin. Ciprofloxacin Etest showed a MIC of 0.38 mg/L (intermediate) for Cases A–E. Cases F–H had been discarded and no further susceptibility testing could be performed. Of note, Case F had been reported to be susceptible to ciprofloxacin by the originating laboratory, based on the CDS method and interpretation (19). This minor difference in categorical interpretation could have been contributed by the ciprofloxacin MIC value of the strain being close to the CLSI M100 (32nd edition) (18) breakpoint of ≤0.25 mg/L. All five isolates were resistant to trimethoprim/sulfamethoxazole (MIC ≥320 mg/L). All isolates available for testing remained susceptible to azithromycin, gentamicin, amikacin, cefepime, ertapenem, and meropenem.

All five of the Singapore isolates carried the genes encoding extended-spectrum beta-lactamase (ESBL) $bla_{\mathrm{CTX-M-15}}$ and $bla_{\mathrm{TEM-1}}$, consistent with the ceftriaxone-resistant ESBL phenotype observed (Figure 4B). Genetic markers for resistance to sulphonamides (*sul2*) and folate synthesis inhibitors (*dfrA14*) were identified. The presence of *qnrS1* could have contributed to the borderline intermediate resistance to ciprofloxacin. No *gyrA*, *gyrB* and *parC* mutations associated with quinolone resistance were detected. Genetic determinants conferring resistance to azithromycin, aminoglycosides and tetracycline were not found. Phenotypic and genotypic AMR profiles were therefore concordant for the panel of antimicrobials tested. We looked for, but did not find other AMR genes of significant public health concern, such as *mcr-1* encoding resistance to colistin (28) or genes encoding carbapenemases (29).

The co-existence of the ESBL genes $bla_{\mathrm{CTX-M-15}}$ and $bla_{\mathrm{TEM-1}}$ in an *S. flexneri* isolate is an uncommon observation. We analyzed 11,143 publicly available *S. flexneri* genomes for the presence of $bla_{\mathrm{CTX-M-15}}$ and $bla_{\mathrm{TEM-1}}$ genes (Figure 4C). Among the 11,143 genomes included in the analysis, 3,665 (32.89%) carry $bla_{\mathrm{TEM-1}}$ and 238 (2.14%) carry $bla_{\mathrm{CTX-M-15}}$. There were 95 (0.85%) *S. flexneri* genomes known to be carrying both $bla_{\mathrm{CTX-M-15}}$ and $bla_{\mathrm{TEM-1}}$ globally. Out of these 95 ESBL *S. flexneri* genomes, 86 (90.53%) were reported from three high-resource locations, namely the United Kingdom ($n = 46$), Ireland ($n = 3$), and the USA ($n = 37$). Three of the remaining nine ESBL genomes were reported from Bangladesh ($n = 1$), Kuwait ($n = 1$), and Pakistan ($n = 1$). The remainder of the genomes ($n = 6$) did not have associated geographical location data available for analysis. Four of these $bla_{\mathrm{CTX-M-15}}$- and $bla_{\mathrm{TEM-1}}$-carrying genomes

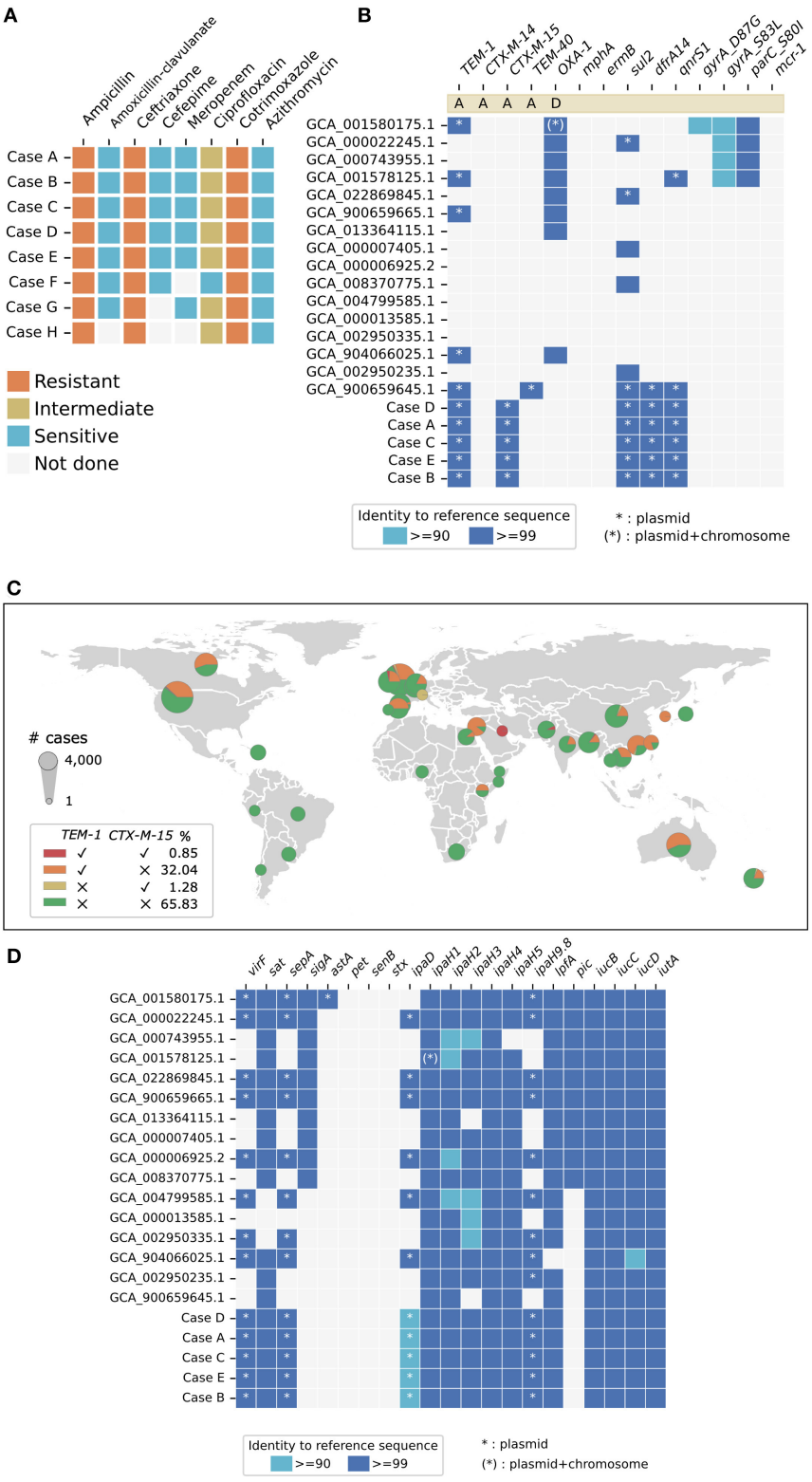

FIGURE 4
Summary of antimicrobial resistance and virulence markers. **(A)** Cases A–E had identical phenotypic antimicrobial susceptibility profiles. All isolates were resistant to ceftriaxone and had intermediate resistance to ciprofloxacin. The antibiograms for Case F–H were retrieved from the

(Continued)

FIGURE 4
laboratory information systems of Hospital III (Case F) and Hospital V (Case G and H), as the isolates had been discarded and were not available for sequencing. **(B)** Cases A–E had identical genotypic antimicrobial susceptibility profiles and contained *blaCTX-M-15* and *blaTEM-1*, consistent with the extended-spectrum beta-lactamases (ESBL) phenotype observed. **(C)** Global distribution of *blaTEM-1* and/or *blaCTX-M-15* containing *Shigella flexneri* are based on publicly available data (NCBI Pathogen Detection as of 24 April 2022). 0.85% of all available *S. flexneri* genomes (n = 11134) contain both *blaCTX-M-15* and *blaTEM-1*. **(D)** Cases A–E had identical genetic markers for a panel of virulence factors, of which *virF*, *sepA*, *astA*, and *ipaH9.8* were encoded on plasmids. Sixteen complete *S. flexneri* reference genomes were included for comparison.

(GCA_013457635.1, GCA_013457815.1, GCA_013457355.10, and GCA_013455205.1) were the same genomes identified to have ≥99.99% ANI and cluster most closely with the Singapore *S. flexneri* isolate (Figure 3C).

In silico whole-genome sequence analysis identified the same list of chromosomal and plasmid-mediated virulence genes in all five of the Singapore *S. flexneri* isolates (Figure 4D). *VirF*, encoding the master activator necessary for invasion and pathogenicity (30), was found on the large 220kb virulence plasmid, pINV. The virulence gene *ipaH9.8* was found on the plasmid, while *ipaH1*, *ipaH2*, *ipaH3*, *ipaH4* and *ipaH5* were found on the chromosome. The genomes also possess virulence genes responsible for adhesion and cytotoxicity, namely, long polar fimbriae (*lpfA*), secreted autotransporter toxin (*sat*), *Shigella* extracellular protein A (*sepA*). Virulence genes related to enterotoxin production, such as *sigA*, *pet*, *senB*, and *stx*, were notably absent in these isolates. Sixteen complete *S. flexneri* genomes downloaded from NCBI which were bioinformatically confirmed to be *S. flexneri* (found to contain genetic marker(s) for *ipaH* but not genetic marker for *lacY*) were included in the analysis. Among these downloaded genomes, eight genomes notably did not contain *virF*, and seven and nine genomes did not contain *ipaH9.8* and *ipaD*, respectively. The lack of consistent detection of these virulence plasmid-mediate genes suggest that existing complete genome data available on NCBI may not reliably detect plasmid-mediate genes. This inconsistency could be due to the loss of the virulence plasmid due to processes related to laboratory handling, or due to limitations related to short-read whole genome sequencing and genome assembly. Nonetheless, due to the limited number of confirmed *S. flexneri* complete genomes (n = 16) available, we included all sixteen genomes in the analysis to capture the diversity found in all available data.

Discussion

We report here the rapid detection of a cluster of clonal MDR *S. flexneri* in Singapore, and the first report of MDR *S. flexneri* serotype 1c (7a), identified using a laboratory-based sentinel surveillance model. Sentinel sequence-based surveillance integrates lower-cost near real-time sequence-based surveillance with routine diagnostic workflow, enabling early detection of possible clonal clusters. The rapid generation of sequence data is particularly advantageous for pathogens and diseases not routinely monitored by public health agencies, as the detection of such outbreaks is likely to be challenging. Curated, portable sequence data generated from sentinel surveillance sites may provide resources for early alerts for public health investigations and interventions.

We demonstrated the clonality of *S. flexneri* isolates in several ways. Firstly, the genomes under investigation were compared to 16 other complete reference *S. flexneri* genomes. Phylogenetic analysis showed that all five genomes belong to the same phylogenetic branch, with a maximum of 20 core genome SNP difference among them (Figure 3A). Secondly, phylogenetic analysis was performed for the five genomes under investigation and 200 publicly available *S. flexneri* whole-genome sequences which were found to be most similar to the query genomes (ANI ≥99.99%). The five genomes under investigation belong to the same distinct phylogenetic branch (Figure 3C). Thirdly, *in silico* typing was performed using ShigaTyper and the Enterobase *Escherichia/Shigella* MLST scheme, and all five isolates were found to belong to serotype 1c (7a) and the ST245 clonal complex, respectively (Figure 3B). Lastly, analysis of AMR and virulence genes showed that all five isolates had identical AMR and virulence gene profiles, which were found to differ from the most closely related publicly available genomes (Figures 4B,D).

This distinct cluster of MDR *S. flexneri* serotype 1c (7a) isolates thus most likely represents a previously unreported clonal source of *S. flexneri* infection. *Shigella flexneri* serotype 1c (7a) was first described in Bangladesh in 1988 (31) and then became widely reported in multiple geographical regions (8, 32–34). Subsequent core genes phylogenetic analysis suggested that there may be two distinct *S. flexneri* 1c lineages, one which originated from ancestral serotype 1a and the other from ancestral serotype 1b (35). Previously reported *S. flexneri* 1c isolates were found to harbor multiple AMR genes, including various extended-spectrum beta-lactamases (35, 36). *Shigella flexneri* serotype 1c (7a) containing $bla_{\mathrm{CTX-M-15}}$ and $bla_{\mathrm{TEM-1}}$, however, remains relatively uncommon based on 11,143 publicly available genomes included in our analysis (Figure 4C). To our knowledge, there have been no prior reports of clonal outbreaks caused by *S. flexneri* serotype 1c (7a) containing $bla_{\mathrm{CTX-M-15}}$ and $bla_{\mathrm{TEM-1}}$.

The rise in AMR in *S. flexneri* is a public health concern. Current therapeutic guidelines advocate azithromycin, ciprofloxacin or ceftriaxone as first-line therapies, and

trimethoprim-sulfamethoxazole or ampicillin as second-line therapies if the isolates are tested susceptible (37). Ciprofloxacin, however, should only be used if the MIC is lower than 0.12 μg/ml, as MIC 0.12 μg/ml or higher may be associated with the presence of a quinolone resistance gene (38). The ciprofloxacin MIC for the isolates described in this study ranged from 0.25 to 0.38 μg/mL, making ciprofloxacin an unfavorable therapeutic option despite the categorical interpretations based on CLSI M100 (18). Therefore, the isolates described in this study cannot be optimally treated with four of the five recommended antibiotics (ampicillin, trimethoprim-sulfamethoxazole, ceftriaxone, and ciprofloxacin), leaving azithromycin as the only therapeutic option.

With increasing reports of MDR and azithromycin-resistant *S. flexneri* (33, 39–45), as well as extensively drug-resistant *S. sonnei* (46, 47), early detection of shigellosis outbreaks carries both therapeutic and public health importance. Current genome databases such as NCBI Pathogen Detection (https://www.ncbi.nlm.nih.gov/pathogens/) facilitate outbreak investigation and resolution by matching pathogen genomes from diverse sources and geographical locations (48). However, the success of this approach is dependent on the availability of genome sequence data from all domains within the One Health framework (49), as well as meticulous standardization of whole-genome sequence data and accompanying metadata, which enables accurate comparisons and source-tracking. In this study, we analyzed 11,134 publicly available *S. flexneri* genomes and 16 complete reference genomes along with our isolates. None of the genomes clustered with our isolates based on our phylogenetic analysis. The most closely related genomes (Figure 3C) were obtained from human clinical samples in the United Kingdom, which were sequenced as part of surveillance. No additional data were available for further investigation or source tracking.

There are several limitations to this study. Firstly, no attempt was made to collect epidemiological or clinical data. Epidemiological and clinical investigations are important parts of traditional outbreak investigations. However, the sentinel sequence-based surveillance model we implemented focused on objective whole-genome sequence data, which is less prone to subjective interpretations, such as patients' recall of dietary history. The outbreak isolate sequence and relatedness data alone are sufficient to constitute an alert for further epidemiological and public health investigations by the appropriate agencies. Secondly, the sentinel site did not serve all hospitals in Singapore. The sentinel site routinely receives *Shigella* isolates from three district hospitals and two community hospitals. Nonetheless, we were able to analyze five isolates from two geographically distinct district hospitals, as well as obtained information on additional cases from two other hospitals. Thirdly, we used the nanopore platform for rapid whole-genome sequencing. A major criticism of the nanopore platform has been its base-level accuracy. We have mitigated this risk by achieving a high sequencing depth (>100× coverage), as well as using the super high accuracy basecaller and polishing the draft genome.

In conclusion, we were able to detect a clonal cluster of MDR *S. flexneri* near real-time by sentinel sequence-based surveillance. Compared to the traditional central reference laboratory structure, this strategy saved time and resources by extending existing routine laboratory workflows. We were able to alert the public health agency of a possible clonal cluster within an actionable timeframe. As jurisdictions work to improve pandemic preparedness, considerations should be made to develop a network and infrastructure for sentinel sequence-based surveillance, which has the flexibility to be rapidly deployed during sporadic outbreaks, epidemics, and pandemics (50).

Data availability statement

The original contributions presented in the study are included in the article/Supplementary materials, further inquiries can be directed to the corresponding author. The genome data of the five *S. flexneri* isolates were submitted to the GenBank database under the BioProject ID PRJNA841078, with BioSample accessions SAMN28576597, SAMN28576598, SAMN28576599, SAMN28576600, and SAMN28576601.

Author contributions

KK and CS conceptualized, designed, and planned the project. JChu, KL, WH, ST, and DC planned and performed wet-lab experiments with KK's and CS's supervision. HY performed computational analysis with CS's supervision. KG, ST, TN, MM, JChi, YT, JS, and TK provided clinical samples and clinical expert opinion. KK, HY, and CS wrote the manuscript with inputs from all authors. NN provided feedback on computational and wet-lab experiments, as well as manuscript. All authors contributed to the article and approved the submitted version.

Funding

KK is supported by the Singapore National Medical Research Council Research Training Fellowship. This work is supported by funding's from Singapore General Hospital, Chiang Mai University and A*STAR.

Acknowledgments

We thank the staff of Singapore General Hospital Diagnostic Bacteriology Laboratory for the technical support.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.964640/full#supplementary-material

References

1. Khalil IA, Troeger C, Blacker BF, Rao PC, Brown A, Atherly DE, et al. Morbidity and mortality due to *Shigella* and enterotoxigenic *Escherichia coli* diarrhoea: the Global Burden of Disease Study 1990-2016. *Lancet Infect Dis.* (2018) 18:1229–40. doi: 10.1016/S1473-3099(18)30475-4

2. DuPont HL, Levine MM, Hornick RB, Formal SB. Inoculum size in shigellosis and implications for expected mode of transmission. *J Infect Dis.* (1989) 159:1126–8. doi: 10.1093/infdis/159.6.1126

3. Khalil I, Troeger CE, Blacker BF, Reiner RC Jr. Capturing the true burden of *Shigella* and ETEC: the way forward. *Vaccine.* (2019) 37:4784–6. doi: 10.1016/j.vaccine.2019.01.031

4. Anderson JD. 4th, Bagamian KH, Muhib F, Amaya MP, Laytner LA, Wierzba T, Rheingans R. Burden of enterotoxigenic *Escherichia coli* and *Shigella* non-fatal diarrhoeal infections in 79 low-income and lower middle-income countries: a modelling analysis. *Lancet Glob Health.* (2019) 7:e321–30. doi: 10.1016/S2214-109X(18)30483-2

5. Kotloff KL, Winickoff JP, Ivanoff B, Clemens JD, Swerdlow DL, Sansonetti PJ, et al. Global burden of *Shigella* infections: implications for vaccine development and implementation of control strategies. *Bull World Health Organ.* (1999) 77:651–66.

6. Livio S, Strockbine NA, Panchalingam S, Tennant SM, Barry EM, Marohn ME, et al. *Shigella* isolates from the global enteric multicenter study inform vaccine development. *Clin Infect Dis.* (2014) 59:933–41. doi: 10.1093/cid/ciu468

7. Chang Z, Zhang J, Ran L, Sun J, Liu F, Luo L, et al. The changing epidemiology of bacillary dysentery and characteristics of antimicrobial resistance of *Shigella* isolated in China from 2004-2014. *BMC Infect Dis.* (2016) 16:685. doi: 10.1186/s12879-016-1977-1

8. von Seidlein L, Kim DR, Ali M, Lee H, Wang X, Thiem VD, et al. A multicentre study of *Shigella* diarrhoea in six Asian countries: disease burden, clinical manifestations, and microbiology. *PLoS Med.* (2006) 3:e353. doi: 10.1371/journal.pmed.0030353

9. Kotloff KL, Riddle MS, Platts-Mills JA, Pavlinac P, Zaidi AKM. Shigellosis. *Lancet.* (2018) 391:801–12. doi: 10.1016/S0140-6736(17)33296-8

10. Chung The H, Bodhidatta L, Pham DT, Mason CJ, Ha Thanh T, Voong Vinh P, et al. Evolutionary histories and antimicrobial resistance in *Shigella flexneri* and *Shigella sonnei* in Southeast Asia. *Commun Biol.* (2021) 4:353. doi: 10.1038/s42003-021-01905-9

11. *WHO publishes list of bacteria for which new antibiotics are urgently needed*. Available online at: https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed (accessed June 24, 2022).

12. Baker KS, Dallman TJ, Ashton PM, Day M, Hughes G, Crook PD, et al. Intercontinental dissemination of azithromycin-resistant shigellosis through sexual transmission: a cross-sectional study. *Lancet Infect Dis.* (2015) 15:913–21. doi: 10.1016/S1473-3099(15)00002-X

13. Public Health England. *Shigella: guidance, data and analysis*. Gov.uk (2013). Available online at: https://www.gov.uk/government/collections/shigella-guidance-data-and-analysis (accessed April 25, 2022).

14. Ministry Of Health - CDLENS. Available online at: https://www.cdlens.moh.gov.sg/cdlens/ (Accessed April 25, 2022).

15. Nygren BL, Schilling KA, Blanton EM, Silk BJ, Cole DJ, Mintz ED. Foodborne outbreaks of shigellosis in the USA, 1998-2008. *Epidemiol Infect.* (2013) 141:233–41. doi: 10.1017/S0950268812000222

16. McLarty K, Paranthaman K, Jenkins C, Sedgwick J, Crawley-Boevey E. Lessons learned from the investigation and management of an outbreak of *Shigella flexneri* associated with a restaurant in London, 2019-2020. *Public Health.* (2022) 205:130–2. doi: 10.1016/j.puhe.2022.01.036

17. Strockbine NA, Bopp CA, Fields PI, Kaper JB, Nataro JP. *Escherichia*, *Shigella*, and *Salmonella*. In: *Manual of Clinical Microbiology*. Washington, DC: ASM Press (2015).

18. M100. *Clinical & Laboratory Standards Institute*. Available online at: https://clsi.org/standards/products/microbiology/documents/m100/ (accessed April 26, 2022).

19. The CDS Antibiotic Susceptibility Test. Available online at: http://cdstest.net/ (accessed April 26, 2022).

20. Kolmogorov M, Yuan J, Lin Y, Pevzner PA. Assembly of long, error-prone reads using repeat graphs. *Nat Biotechnol.* (2019) 37:540–6. doi: 10.1038/s41587-019-0072-8

21. Treangen TJ, Ondov BD, Koren S, Phillippy AM. The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. *Genome Biol.* (2014) 15:524. doi: 10.1186/s13059-014-0524-x

22. Download. Available online at: https://www.ncbi.nlm.nih.gov/home/download/ (accessed May 24, 2022).

23. Bruen TC, Philippe H, Bryant D. A simple and robust statistical test for detecting the presence of recombination. *Genetics.* (2006) 172:2665–81. doi: 10.1534/genetics.105.048975

24. Kurtz S, Phillippy A, Delcher AL, Smoot M, Shumway M, Antonescu C, et al. Versatile and open software for comparing large genomes. *Genome Biol.* (2004) 5:R12. doi: 10.1186/gb-2004-5-2-r12

25. Wu Y, Lau HK, Lee T, Lau DK, Payne J. *In silico* serotyping based on whole-genome sequencing improves the accuracy of *Shigella* identification. *Appl Environ Microbiol.* (2019) 85:e00165-19. doi: 10.1128/AEM.00165-19

26. National Database of Antibiotic Resistant Organisms (NDARO). Available online at: https://www.ncbi.nlm.nih.gov/pathogens/antimicrobial-resistance/ (accessed May 9, 2022).

27. BioSample. Available online at: https://www.ncbi.nlm.nih.gov/biosample/10934589 (accessed May 9, 2022).

28. Liang B, Roberts AP, Xu X, Yang C, Yang X, Wang J, et al. Transferable plasmid-borne mcr-1 in a colistin-resistant *Shigella flexneri* isolate. *Appl Environ Microbiol.* (2018) 84:e02655-17. doi: 10.1128/AEM.02655-17

29. Thamizhmani R, Rhagavan R, Sugunan AP, Vijayachari P. VIM- and IMP-type metallo-β-lactamase-producing *Shigella* spp. in childhood diarrhea from Andaman Islands. *Infect Dis.* (2015) 47:749–50. doi: 10.3109/23744235.2015.1022874

30. Di Martino ML, Falconi M, Micheli G, Colonna B, Prosseda G. The multifaceted activity of the VirF regulatory protein in the *Shigella* lifestyle. *Front Mol Biosci.* (2016) 3:61. doi: 10.3389/fmolb.2016.00061

31. Wehler T, Carlin NI. Structural and immunochemical studies of the lipopolysaccharide from a new provisional serotype of *Shigella flexneri*. *Eur J Biochem.* (1988) 176:471–6. doi: 10.1111/j.1432-1033.1988.tb14304.x

32. Ahmed SF, Klena J, Husain T, Monestersky J, Naguib A, Wasfy MO. Genetic characterization of antimicrobial resistance of *Shigella flexneri* 1c isolates from patients in Egypt and Pakistan. *Ann Clin Microbiol Antimicrob.* (2013) 12:9. doi: 10.1186/1476-0711-12-9

33. Qiu S, Xu X, Wang Y, Yang G, Wang Z, Wang H, et al. Emergence of resistance to fluoroquinolones and third-generation cephalosporins in *Shigella flexneri* subserotype 1c isolates from China. *Clin Microbiol Infect.* (2012) 18:E95–8. doi: 10.1111/j.1469-0691.2012.03768.x

34. Stagg RM, Cam PD, Verma NK. Identification of newly recognized serotype 1c as the most prevalent *Shigella flexneri* serotype in northern rural Vietnam. *Epidemiol Infect.* (2008) 136:1134–40. doi: 10.1017/S0950268807009600

35. Parajuli P, Minh BQ, Verma NK. Newly emerged serotype 1c of *Shigella flexneri*: multiple origins and changing drug resistance landscape. *Genes (Basel).* (2020) 11:1042. doi: 10.3390/genes11091042

36. Muthuirulandi Sethuvel DP, Devanga Ragupathi NK, Anandan S, Veeraraghavan B. Update on: *Shigella* new serogroups/serotypes and their antimicrobial resistance. *Lett Appl Microbiol.* (2017) 64:8–18. doi: 10.1111/lam.12690

37. Shane AL, Mody RK, Crump JA, Tarr PI, Steiner TS, Kotloff K, et al. 2017 Infectious diseases society of America clinical practice guidelines for the diagnosis and management of infectious diarrhea. *Clin Infect Dis.* (2017) 65:e45–80. doi: 10.1093/cid/cix669

38. HAN Archive - 00401. (2018). Available online at: https://emergency.cdc.gov/han/han00401.asp (accessed May 18, 2022).

39. Guglielmino CJD, Kakkanat A, Forde BM, Rubenach S, Merone L, Stafford R, et al. Outbreak of multi-drug-resistant (MDR) *Shigella flexneri* in northern Australia due to an endemic regional clone acquiring an IncFII plasmid. *Eur J Clin Microbiol Infect Dis.* (2021) 40:279–86. doi: 10.1007/s10096-020-04029-w

40. Mitchell HD, Thomson NR, Jenkins C, Dallman TJ, Painset A, Kirwan P, et al. Linkage of whole genome sequencing, epidemiological, and clinical data to understand the genetic diversity and clinical outcomes of *Shigella flexneri* among men who have sex with men in England. *Microbiol Spectr.* (2021) 9:e0121321. doi: 10.1128/Spectrum.01213-21

41. Qiu S, Wang Y, Xu X, Li P, Hao R, Yang C, et al. Multidrug-resistant atypical variants of *Shigella flexneri* in China. *Emerg Infect Dis.* (2013) 19:1147–50. doi: 10.3201/eid1907.121221

42. Moreno-Mingorance A, Espinal P, Rodriguez V, Goterris L, Fàbrega A, Serra-Pladevall J, et al. Circulation of multi-drug-resistant *Shigella sonnei* and *Shigella flexneri* among men who have sex with men in Barcelona, Spain, 2015-2019. *Int J Antimicrob Agents.* (2021) 58:106378. doi: 10.1016/j.ijantimicag.2021.106378

43. Logan NZ, Karp BE, Tagg KA, Burns-Lynch C, Chen J, Garcia-Williams A, et al. 130. increase in multidrug resistance (2011–2018) and the emergence of extensive drug resistance (2020) in *Shigella sonnei* in the United States. *Open Forum Infect Dis.* (2020) 7:S195. doi: 10.1093/ofid/ofaa439.440

44. Farajzadeh Sheikh A, Moradi Bandbal M, Saki M. Emergence of multidrug-resistant *Shigella* species harboring extended-spectrum beta-lactamase genes in pediatric patients with diarrhea from southwest of Iran. *Mol Biol Rep.* (2020) 47:7097–106. doi: 10.1007/s11033-020-05776-x

45. Sheikh AF, Moosavian M, Abdi M, Heidary M, Shahi F, Jomehzadeh N, et al. Prevalence and antimicrobial resistance of *Shigella* species isolated from diarrheal patients in Ahvaz, southwest Iran. *Infect Drug Resist.* (2019) 12:249–53. doi: 10.2147/IDR.S187861

46. Williamson D, Ingle D, Howden B. Extensively drug-resistant Shigellosis in Australia among men who have sex with men. *N Engl J Med.* (2019) 381:2477–9. doi: 10.1056/NEJMc1910648

47. Extensively drug-resistant *Shigella sonnei infections* - Europe. Available online at: https://www.who.int/emergencies/disease-outbreak-news/item/extensively-drug-resistant-shigella-sonnei-infections---europe (accessed May 18, 2022).

48. Success Stories. Avaialble online at: https://www.ncbi.nlm.nih.gov/pathogens/success_stories/ (accessed May 18, 2022).

49. One Health. Available online at: https://www.who.int/news-room/questions-and-answers/item/one-health (accessed May 18, 2022).

50. Ko KKK, Chng KR, Nagarajan N. Metagenomics-enabled microbial surveillance. *Nat Microbiol.* (2022) 7:486–96. doi: 10.1038/s41564-022-01089-w

frontiers | Frontiers in Public Health

TYPE Original Research
PUBLISHED 04 August 2022
DOI 10.3389/fpubh.2022.975720

OPEN ACCESS

EDITED BY
Amir Abdoli,
Jahrom University of Medical Sciences, Iran

REVIEWED BY
Sandrama Nadan,
National Institute of Communicable Diseases (NICD), South Africa
Mohamad S. Hakim,
Gadjah Mada University, Indonesia
Haibo Wang,
Zhuhai International Travel Healthcare Center, China
Alpha Fardah Athiyyah,
Airlangga University, Indonesia

*CORRESPONDENCE
Peng Liu
ammsliupeng@163.com
Qingyu Lv
lvqingyu2004@126.com
Hua Jiang
jhua76@126.com

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Infectious Diseases – Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

RECEIVED 22 June 2022
ACCEPTED 19 July 2022
PUBLISHED 04 August 2022

CITATION
Wang Y, Zheng Y, Li Y, Zhang S, Wang X, Zong H, Huang W, Kong D, Jiang Y, Liu P, Lv Q and Jiang H (2022) Development of a rapid homogeneous immunoassay for detection of rotavirus in stool samples. *Front. Public Health* 10:975720. doi: 10.3389/fpubh.2022.975720

Development of a rapid homogeneous immunoassay for detection of rotavirus in stool samples

Ye Wang[1,2†], Yuling Zheng[2†], Yan Li[3], Shengwei Zhang[2,4], Xin Wang[3], Huijun Zong[2,3], Wenhua Huang[2], Decong Kong[2], Yongqiang Jiang[2], Peng Liu[2*], Qingyu Lv[2*] and Hua Jiang[2*]

[1]College of Life Science and Technology, Beijing University of Chemical Technology, Beijing, China, [2]State Key Laboratory of Pathogen and Biosecurity, Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences (AMMS), Beijing, China, [3]The Fifth Medical Center of PLA General Hospital, Beijing, China, [4]Department of Clinical Laboratory, Dongfang Hospital, Beijing University of Chinese Medicine, Beijing, China

Rotavirus is the main pathogen causing acute viral gastroenteritis. Accurate and rapid diagnosis of rotavirus infection is important to determine appropriate treatment, prevention of unnecessary antibiotics use and control of infection spread. In this study, we established a rapid, accurate, and sensitive amplified luminescent proximity homogeneous assay linked immunosorbent assay (AlphaLISA) for detecting rotavirus and evaluated its efficacy in human stool samples. Our results demonstrated that the sensitivity of AlphaLISA (5^{-8}) significantly exceeded that of the immunochromatographic assay (ICA, 5^{-4}) for rotavirus antigen detection. The intra-assay and inter-assay coefficients of variation were 2.99–3.85% and 5.27–6.51%, respectively. Furthermore, AlphaLISA was specific for rotavirus and did not cross-react with other common diarrhea viruses. AlphaLISA and real-time reverse transcription polymerase chain reaction (RT-qPCR, which is considered a gold standard for detecting diarrhea viruses) tests showed consistent results on 235 stool samples, with an overall consistency rate of 97.87% and a kappa value of 0.894 ($P < 0.001$). The overall consistency rate of ICA compared with RT-qPCR was 95.74%. AlphaLISA showed better consistency with RT-qPCR than the routinely used ICA for rotavirus detection in stool samples. The AlphaLISA method can be used in clinical practice for the rapid, accurate, and sensitive detection of rotavirus infection.

KEYWORDS

diarrhea, rotavirus, detection, AlphaLISA, homogeneous assay

Introduction

Acute gastroenteritis (AGE) is a relatively common infectious disease that affects hundreds of millions of people worldwide every year, especially in low-income countries. It is one of the leading causes of illness and death in children under 5 years of age (1, 2). Rotavirus is the major cause of acute viral gastroenteritis in infants and young children,

which is transmitted primarily *via* the fecal-oral route (3). Watery diarrhea, vomiting, headache, fever, and stomachic abdominal cramps are all clinical symptoms of rotavirus illness (4). Rotavirus infection can cause asymptomatic or mild diarrhea in adults, but immunocompromised individuals are particularly susceptible to infection and can suffer from severe diarrhea (5). Patients with gastroenteritis are primarily treated with oral drugs or intravenous fluids. Viral gastroenteritis is usually not treated with antibiotics (6), so accurate and rapid identification of gastroenteritis pathogens could help reduce unnecessary antibiotic use.

Numerous techniques can be used for rotavirus detection, including traditional detection methods, immunological detection methods, and molecular biological detection methods. Traditional detection methods such as virus isolation in cell culture, electron microscopy, and serological tests are difficult and lengthy to operate (7). Immunological detection methods include enzyme-linked immunosorbent assay (ELISA) technology and immunochromatographic assay (ICA) to detect pathogens through the specific binding of antibodies and antigens. ELISA requires multiple washing steps to remove nonspecifically bound reactants, which is time-consuming (8). The immunochromatographic assay can give detection results in a short time, but has low sensitivity (9). Molecular biological methods such as real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) are highly sensitive and specific, but they require specialized techniques and equipment and take a lot of time, which is not conducive to rapid detection and large-scale screening (10).

This study developed a rotavirus detection method based on the amplified luminescent proximity homogeneous assay linked immunosorbent assay (AlphaLISA), which primarily depends on the interaction between donor microspheres and acceptor microspheres. The surface of donor microspheres has been labeled with streptavidin, which can capture biotinylated antibodies. The acceptor microspheres are then conjugated with detection antibodies. The two microspheres were close when the test antigen was bound to the specific antibody. The photosensitizer on the donor emitted singlet oxygen molecules if irradiated by a 680 nm laser, and the singlet oxygen molecules proliferate and reach the surface of acceptor beads in the proximity of 200 nm. This triggers a chemical reaction and generates a chemiluminescence signal at 615 nm on the surface of the acceptor beads (11). The AlphaLISA has many advantages over conventional detection methods: it is easy to operate, highly sensitive, fast, uses less volume of sample, and has become a highly accurate *in-vitro* diagnostic tool. In this study, we evaluate this system for rotavirus detection in stool and compare the efficacy of AlphaLISA with conventional detection methods.

Materials and methods

Stool samples

Two hundred and thirty five stool samples were collected from patients with symptoms of acute gastroenteritis from the Fifth Medical Center of PLA General Hospital and Dongfang Hospital in Beijing, between December 2019 and June 2022. Patient ages ranged from 7 days to 91 years (average, 42.9 years); 26 samples (11.1%) were from patients under 5 years of age and 54 samples (23.0%) were from patients who were 65 years of age or older. The fresh stool samples were aliquoted and frozen immediately at −80°C until they were used for the comparative tests (AlphaLISA, RT-qPCR and ICA). This study was approved by the Institutional Review Board (IRB) of the Fifth Medical Center of PLA General Hospital (reference no. ky-2019-1-4) and Dongfang Hospital (reference no. JDF-IRB-2020003501). All samples were obtained with the patient's consent.

Antigen, antibody, and reagent

Rotavirus antigen (Simian rotavirus SA11) inactivated using gamma irradiation was purchased from Microbix (Toronto, Canada). Rotavirus antibodies 10R-30C and 10R-30E were purchased from Fitzgerald (North Acton, MA, USA). Unconjugated Eu-acceptor beads, streptavidin-coated donor beads, 1/2 AreaPlateTM-96 well plate, and 10× AlphaLISA immunoassay buffer were purchased from PerkinElmer (Waltham, MA, USA). EZ-Link® Sulfo-NHS-LC-Biotinylation Kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA). $NaBH_3CN$ and carboxymethoxylamine were purchased from Sigma (St. Louis, MO, USA).

AlphaLISA test

Three microliter biotin (10 mM) was added to 100 μg of the rotavirus antibody (10R-30E) solution. After 1 h of incubation at room temperature, the excess biotin was removed by a Zeba Spin Desalting Column. Conjugation of the rotavirus antibody (10R-30C) to acceptor beads was performed according to the manufacturer's instructions as previously described (12).

Five milligram stool samples were weighed and placed in 1.5 ml centrifuge tubes 0. 1 ml PBS (pH 7.4) was added to make a 0.5% (w/v) suspension. This

suspension was mixed by vortex and centrifuged at 2,500 g for 5 min. The supernatant was collected for the AlphaLISA testing.

AlphaLISA was performed in a white 1/2 AreaPlate™-96. Acceptor beads and biotin-labeled antibodies were mixed in 20 μl, after which 5 μl sample suspension or rotavirus antigen was added. The 25 μl mixture was incubated at 37°C for 15 min, and then 25 μl of streptavidin donor beads were added. After incubation for an additional 10 min at 37°C, the signal was read by SpectraMax™ I3 (Molecular Devices, Sunnyvale, CA, USA).

To optimize the concentration of biotinylated antibodies, antibodies labeled acceptor beads, and streptavidin-coated donor beads, rotavirus antigen at low, middle or high concentrations were detected with three replicates per group. Signal to noise (S/N) ratios were calculated. To evaluate the sensitivity of AlphaLISA, we first performed a 1:25-fold dilution of rotavirus antigens and then prepared serial 5-fold dilutions for AlphaLISA detection. The cutoff value was defined as the average fluorescence intensity of the negative control group plus three standard deviations.

Repeatability of AlphaLISA was assessed by tests of two levels of antigen concentrations. Intra-assay variation was calculated from the variation of 12 determinations of low and high antigen concentrations ($1{:}5^7$ and $1{:}5^3$ dilution ratio) on the same plate and in the same test. On the other hand, inter-assay variation was calculated by antigen detecting in the same manner once-a-day for three consecutive days. The average measured value, standard deviation (SD), and coefficients of variation (CV) were calculated.

RT-qPCR test

Since most infection from human rotavirus is caused by group A viruses, group A rotavirus nonstructural protein 3 gene was tested by RT-qPCR. The total RNA of stool samples was extracted using a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA). RT-qPCR was performed for each sample using AgPath-ID™ one-step RT-PCR reagents (Thermo Scientific, Waltham, MA, USA). The primers and probe used for detecting human group A rotavirus (Rota-F: ACCATCTWCACRTRACCCTCTATGA; Rota-R: GGTCACATAACGCCCCTATAGC; Rota-P: AGTTAAAAGCTAACACTGTCAAA) (13) were synthesized by Sangon Biotech (Shanghai) Co., Ltd. The reaction was performed on a ViiA™ 7 real-time PCR system (Applied Biosystems, CA, USA). The cycling conditions were 45°C for 10 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 45 s. The result was considered positive when the cycle threshold (Ct) value was ≤ 38 and was considered negative when it was > 38. Fourteen stool samples were re-tested using Rotavirus (Group A) Nucleic Acid Assay Kit (Shanghai Liferiver Biotechnology Co., Ltd.), and reverse transcription, amplification, and detection were conducted according to the manufacturer's instructions.

ICA test

A Rotavirus Antigen Assay Kit (Guangzhou Wondfo Biotechnology Co., Ltd.) was used for the immunochromatographic assay. Ten milligram or 50 μl of stool samples were collected and mixed with the sample diluent. Two to three drops (about 60 μl to 80 μl) of the sample solution were added to the sample loading area. After 10 min, the result was considered positive if one line was observed in the control area (C) and another line was observed in the test area (T).

Statistical analysis

Data are presented as mean ± SD. The student's *t*-test was used to compare two groups. The ROC curve, area under the ROC curve (AUC), cutoff value, and kappa coefficient were calculated using the statistical analysis software SPSS 22.0. $P < 0.05$ was considered statistically significant.

Results

Optimal concentration of biotinylated antibodies, acceptor-conjugated antibody beads, and streptavidin-coated donor beads

Optimal concentrations of biotinylated antibodies would be determined first. The concentration of the biotinylated antibodies varied (0.075, 0.15, and 0.30 μM) while we kept the acceptor-conjugated antibody beads and streptavidin-coated donor beads constant to ensure optimal performance when detecting rotavirus. Results of this assay (Figure 1A) showed that using 0.15 μM of biotinylated antibodies produced the largest S/N ratios, regardless of the antigen concentration. Therefore, 0.15 μM was chosen for subsequent AlphaLISA assay.

The concentration of acceptor beads and streptavidin-coated donor beads was essential for the immunoassay sensitivity and linear range. On the one hand, an excessive amount of chemibeads would provide more opportunities for random collisions between the acceptor beads and donor beads, which increases the background signal and decreases sensitivity. On the other hand, an extremely low amount of chemibeads would decrease the signal and affect the sensitivity of the analysis (14). Therefore, in this assay, 25, 50, and 100 μg/ml antibodies labeled acceptor beads and 20, 40, and 80 μg/ml streptavidin-coated donor beads were separately tested to determine the optimum concentration for the AlphaLISA experiment. After

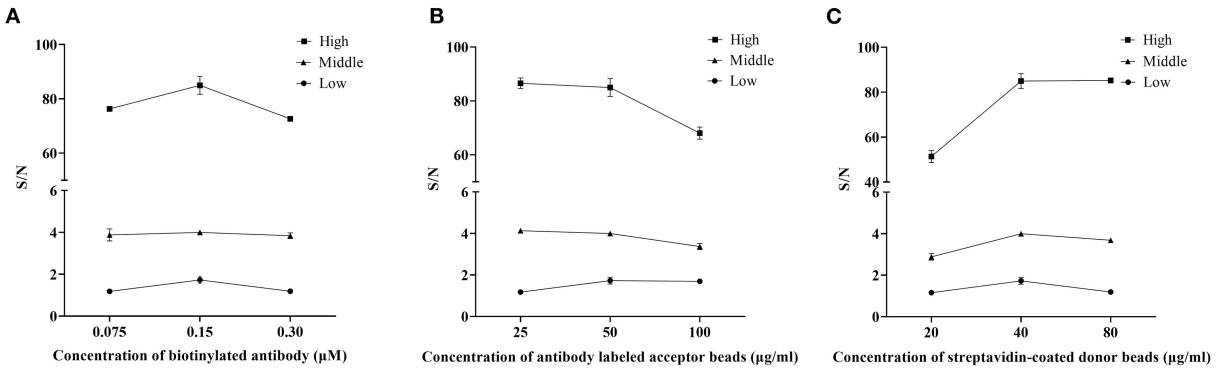

FIGURE 1
Optimization of the AlphaLISA experiment. The effect of a single variable on AlphaLISA S/N was examined by making the other factors constant. **(A)** Effect of the concentration of biotinylated antibodies (μM); **(B)** Effect of the concentration of antibody labeled acceptor beads (μg/ml); **(C)** Effect of the concentration of streptavidin-coated donor beads (μg/ml). Low: the low concentration of rotavirus antigen (dilution ratio: 5^{-7}); Middle: the middle concentration of rotavirus antigen (dilution ratio: 5^{-5}); High: the high concentration of rotavirus antigen (dilution ratio: 5^{-3}). Mean values from 3 trials are plotted, with error bars denoting the standard deviation.

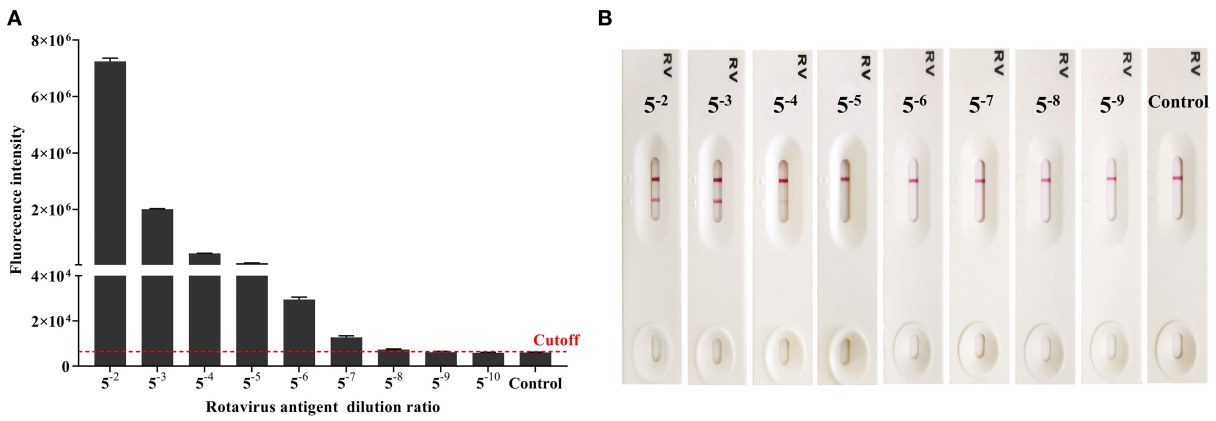

FIGURE 2
Comparison of the detection sensitivity for AlphaLISA and ICA (Result of one experiment shown only, see Supplementary Figure 1 for the others). **(A)** Rotavirus antigen detection using AlphaLISA. Mean values from 3 trials are plotted with error bars denoting the standard deviation; **(B)** Rotavirus antigen detection using the ICA.

considering both S/N and sensitivity, the optimal concentration of acceptor beads was 50 μg/ml (Figure 1B). Similarly, the optimal concentration of streptavidin-coated donor beads was 40 μg/ml (Figure 1C).

Sensitivity and repeatability of detection

Rotavirus antigen was tested using AlphaLISA and ICA, to compared the sensitivity of the two methods. AlphaLISA could detect rotavirus antigens at a dilution of 1: 5^8, whereas ICA could only detect rotavirus antigens in 1: 5^4 dilutions (Figure 2). Therefore, the sensitivity of AlphaLISA significantly exceeded that of ICA. The repeatability experiment showed that, for AlphaLISA, the intra-assay CV was 2.99-3.85%, and the inter-assay CV was 5.27-6.51% (Table 1). This indicates that the AlphaLISA had sufficient repeatability.

Optimization of sample pretreatment conditions

Due to the complexity and heterogeneity of the stool samples, they often must be pretreated to remove interfering substances that might be present. We prepared sample suspensions at different concentrations (1, 0.5, and 0.25%) to evaluate the influence of sample dilution on detection. To avoid false-positive results and ensure a high S/N ratio in the positive

TABLE 1 Intra- and inter-assay coefficients of variation (CV).

Antigen dilution ratio	Intra-assay ($n = 12$)		Inter-assay ($n = 36$)	
	Mean ± SD	CV (%)	Mean ± SD	CV (%)
5^{-7}	12805.25 ± 493.48	3.85	12253.03 ± 797.85	6.51
5^{-3}	1930305.08 ± 57758.57	2.99	1884488.69 ± 99378.86	5.27

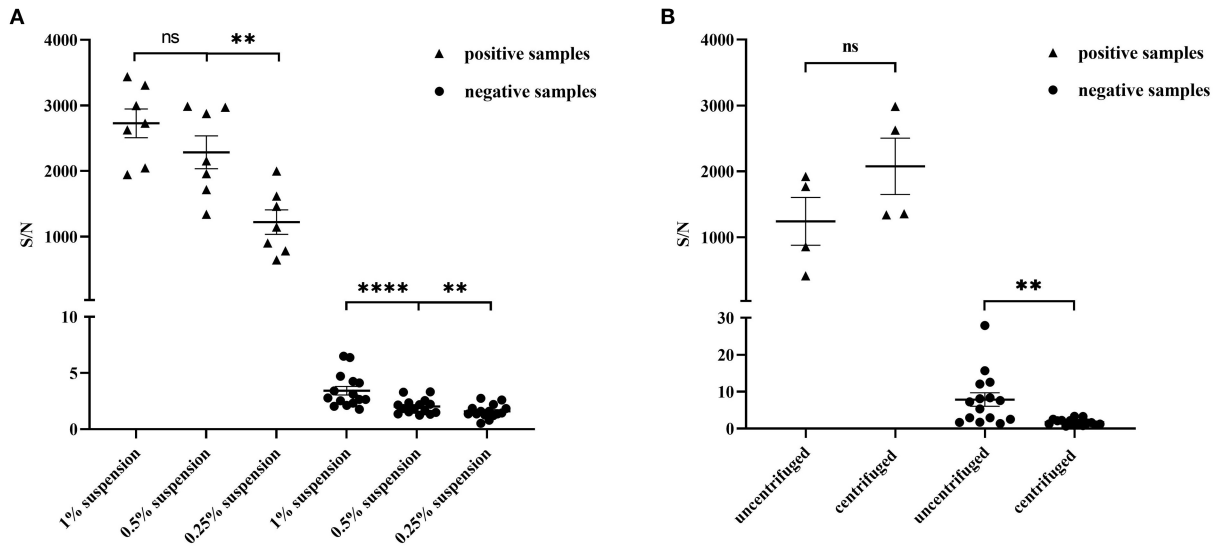

FIGURE 3
Optimization of the stool sample pretreatment for AlphaLISA experiment. **(A)** Influence of 1% suspension, 0.5% suspension and 0.25% suspension on the test results of positive stool samples ($n = 7$) and negative stool samples ($n = 15$); **(B)** Influence of uncentrifuged and centrifuged for positive stool samples ($n = 4$) and negative stool samples ($n = 15$) on detection; $^{**}P < 0.01$; $^{****}P < 0.0001$; ns, not significant.

samples, the optimal concentration of suspension was 0.5% (Figure 3A). We then evaluated the influence of centrifugation treatment on detection and found that it had little effect on the positive samples (Figure 3B). However, for negative samples, the S/N ratio significantly decreased after centrifugation. The treatment reduced the occurrence of false-positive results. The stool samples were diluted into 0.5% (w/v) suspension in the subsequent experiments and centrifuged.

Optimal cutoff value

ROC was plotted based on 235 stool samples, and RT-qPCR is considered the gold standard for detecting rotavirus. The AUC was 0.974 ($P < 0.001$, Figure 4). An AUC > 0.95 typically indicates a very high diagnostic value for a test. Therefore, we chose a cutoff value of 4.9142 on the ROC based on the optimal sensitivity and specificity (Figure 4). Samples with S/N ratio ≥ 4.9142 are considered rotavirus positive, while samples with S/N ratio < 4.9142 are considered rotavirus negative.

Cross-reactivity of AlphaLISA

In addition to rotavirus, other viruses can cause acute gastroenteritis (15–17). For the cross-reactivity evaluation of the detection method used in this study, stool samples with rotavirus, adenovirus, astrovirus, norovirus genogroup I, and norovirus genogroup II were tested using the AlphaLISA method. Triplicate samples and negative controls were set in this assay, and the cutoff value was used to classify them as negative or positive. Results (Figure 5) showed that AlphaLISA could accurately distinguish rotavirus from other diarrhea viruses.

Comparison of AlphaLISA, ICA, and RT-qPCR for detecting rotavirus in clinical stool samples

Two hundred and thirty five stool samples were tested using AlphaLISA, RT-qPCR, and ICA. The comparative results of AlphaLISA, ICA, and RT-qPCR for detecting rotavirus were shown in Table 2. The overall agreement rates between the

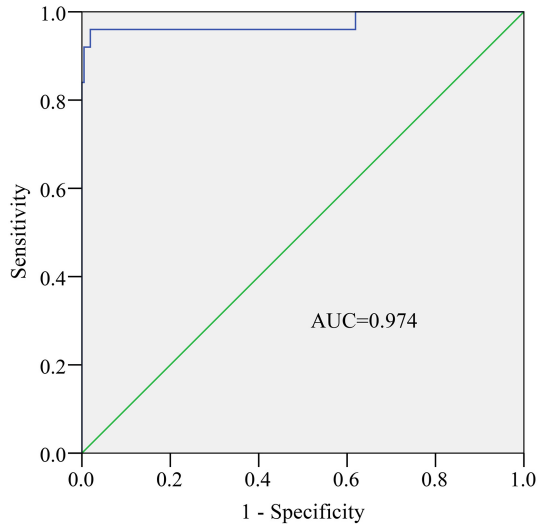

FIGURE 4
Receiver operating characteristic (ROC) curve of AlphaLISA. AUC, area under the curve.

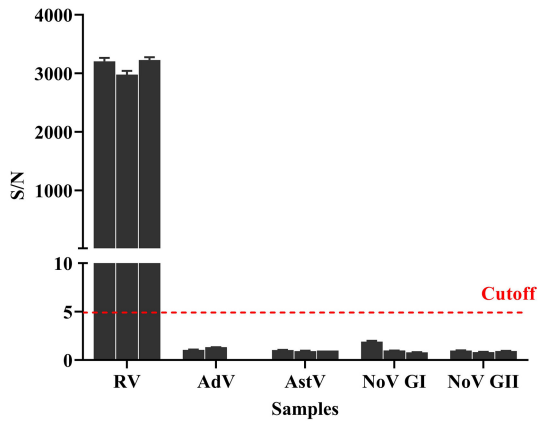

FIGURE 5
Cross-reaction of AlphaLISA. Different diarrhea virus-positive samples (3 for RV, 2 for enteric AdV, 3 for AstV, 3 for NoV GI, and 3 for NoV GII) were analyzed with AlphaLISA. The bar represents the standard deviation. The dotted line represents the cutoff value. RV, rotavirus; AdV, adenovirus; AstV, astrovirus; NoV GI, norovirus genogroup I; NoV GII, norovirus genogroup II.

three methods were 94.04% for rotaviruses. The 14 samples with discordant results in 3 methods were reconfirmed with the commercial PCR kit, and all were consistent with the RT-qPCR results. The overall agreement, positive agreement, and negative agreement of AlphaLISA compared with RT-qPCR were 97.87, 96.00, and 98.10%, respectively. The weighted kappa coefficient was 0.894, and the asymptotic 95% confidence interval was 0.802–0.986. The overall agreement, positive agreement, and negative agreement of ICA compared with RT-qPCR were 95.74, 84.00, and 97.14%, respectively. The weighted kappa coefficient was 0.784, and the asymptotic 95% confidence interval was 0.655–0.913.

Comparison of the AlphaLISA and RT-qPCR tests showed that the results agreed with each other (Kappa > 0.75). However, AlphaLISA yielded four false-positive results (for which RT-qPCR and ICA yielded negative results). This suggested that AlphaLISA might provide false-positive results. One stool sample showed negative results using the AlphaLISA and ICA methods but positive results using RT-qPCR. The Ct value of this sample was 36.89 (Table 3). A low viral load in this sample could be the reason for negative results with AlphaLISA and ICA. In contrast, ICA yielded six false-positive results (RT-qPCR and AlphaLISA yielded negative results) and four false-negative results compared to RT-qPCR, showing that AlphaLISA was more accurate than routine ICA methods for rotavirus detection. Additionally, we found that AlphaLISA was less consistent with ICA, with the weighted kappa coefficient of 0.732 and the asymptotic 95% confidence interval of 0.593-0.871. The overall agreement, positive agreement, and negative agreement of AlphaLISA compared with ICA were 94.47, 77.78, and 96.63%, respectively (Supplementary Table 1).

Discussion

Rotaviruses is the major pathogen cause of acute viral gastroenteritis in infants and young children worldwide, producing a significant disease burden. There is also an extensive literature linking rotavirus to gastroenteritis in adults throughout the world (18). Acute gastroenteritis due to rotavirus can lead to vomiting and watery diarrhea, which in turn causes body fluid loss leading to dehydration and

TABLE 2 Comparison of rotavirus detection results by AlphaLISA, ICA, and RT-qPCR.

Virus		RT-qPCR			
		Positive agreement	Negative agreement	Total agreement	Kappa coefficient (95% CI)
Rotavirus (94.04%, 221/235)*	AlphaLISA	96.00% (24/25)	98.10% (206/210)	97.87% (230/235)	0.894(0.802–0.986)
	ICA	84.00% (21/25)	97.14% (204/210)	95.74% (225/235)	0.784(0.655–0.913)

*Overall agreement rate among three assays.
CI, confidence interval.

TABLE 3 The test results of rotavirus-positive stool samples.

	RT-qPCR		AlphaLISA		ICA	
Sample number	Ct	Results	S/N	Results	Picture	Results
1	16.50	P	2972.18	P		P
2	16.52	P	3228.74	P		P
3	17.14	P	3349.40	P		P
4	17.69	P	2624.43	P		P
5	19.5	P	2874.82	P		P
6	19.88	P	1356.93	P		P
7	19.94	P	2152.41	P		P
8	20.95	P	1957.64	P		P
9	21.22	P	1338.63	P		P
10	21.48	P	2985.35	P		P
11	21.80	P	1714.18	P		P
12	21.97	P	3183.47	P		P
13	22.86	P	2978.86	P		P
14	23.13	P	3206.75	P		P
15	25.91	P	998.82	P		P
16	25.93	P	1011.61	P		P
17	31.73	P	7.49	P		P
18	32.28	P	203.46	P		P
19	32.71	P	161.93	P		P
20	33.01	P	140.19	P		P
21	33.18	P	39.09	P		P
22	34.68	P	14.38	P		N
23	35.06	P	5.14	P		N
24	35.98	P	7.31	P		N
25	36.89	P	1.03	N		N

P, Positive; N, Negative.

affects patient's quality of life seriously. Although the rotavirus incidence has dramatically declined with vaccination among high-income countries, the number of diarrhea and rotavirus deaths remains high in low-income populations with poor access to safe water, sanitation, and urgent medical care in developing countries (1–3). Hence, in order to enable rapid confirmation of acute gastroenteritis pathogens, a rapid and sensitive detection method is still a major concern for monitoring rotavirus outbreaks.

AlphaLISA is a homogeneous immunoassay with high sensitivity which have no need for any separation/washing steps but 5 μl sample and a two-step mixed reaction for detection (19–21). Currently, AlphaLISA has been used for the detection of a wide variety of analytes from proteins to peptides and other small molecules. And it has been extensively used for the detection of several infectious viruses, namely, Hepatitis B virus in human serum and African swine fever virus in porcine serum with high sensitivity and specificity (19, 22). In this study, AlphaLISA method was developed for rapid and sensitive detection of rotavirus.

Numerous AlphaLISA assays have been reported in a variety of sample types ranging from cell lysates (23, 24), to serum (25, 26), to food (12, 27). In this study, we showed, for the first time, the applicability of the AlphaLISA technology for the detection of stool samples. The stool suspension samples were pretreated by dilution and centrifugation to remove interfering substances that might be present, and non-specific reactions of the AlphaLISA assay had been greatly reduced. The AlphaLISA method could detect rotavirus well in stool samples.

The sensitivity and specificity of AlphaLISA in detecting rotavirus was lower than the RT-qPCR method, since the molecular biological methods were considered more sensitive than the immunological method and the RT-qPCR test is considered a gold standard for detecting diarrhea viruses (28, 29). For patients with acute gastroenteritis, the RT-qPCR method is time-consuming as it requires 3 to 4 h to conduct and get the test results. AlphaLISA was a rapid and homogeneous immunoassay which could test rotavirus in 30 min. It could be used as a novel potential on-site rapid detection method and showed better consistency with RT-qPCR than routine ICA methods for rotavirus detection. Compared to convenient operation of ICA, AlphaLISA method still requires manual operation and specific laboratory instruments. But AlphaLISA method is performed according to simple "mix-and-measure" protocols, which is ideally suited for miniaturization and automation. Miniaturization, automated instrumentation will enable this method to be used for point-of-care-testing (POCT) of rotavirus infection. In addition, the method can be used to test up to 384 samples simultaneously by using 384-well plates to increase throughput, reduce reagent consumption. Using portable instruments and reducing reagent costs will facilitate the commercialization and wide application of AlphaLISA for rotavirus detection. Before that larger-scale and multicenter clinical specimens test should be conducted to further validate the commercial utility of AlphaLISA.

In conclusion, the AlphaLISA method developed in this study have high sensitivity and specificity in detection of rotavirus, with short turnaround time (30 min), high reproducibility, and high consistence of detection results to the RT-qPCR method. Therefore, AlphaLISA could be a useful screening tool for rapidly and accurately diagnosing rotavirus infection during viral outbreaks.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author/s.

Ethics statement

The studies involving human participants were reviewed and approved by the Institutional Review Board (IRB) of the Fifth Medical Center of PLA General Hospital (reference no. ky-2019-1-4) and Dongfang Hospital (reference no. JDF-IRB-2020003501). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

PL, QL, and HJ conceived and designed the study and participated in the review and editing of the manuscript. YW and YZ carried out the experimental work and analyzed the data. YW, QL, and HJ wrote the original draft of the manuscript. All authors participated in interpreting the results, read, and approved the final version of this manuscript.

Funding

This work was supported by research grants from the Chinese State Key Project Specialized for Infectious Disease (Grant No. 2018ZX10711001-003-001).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2022.975720/full#supplementary-material

References

1. GBD Diarrhoeal Diseases Collaborator. Estimates of global, regional, national morbidity, mortality, and aetiologies of diarrhoeal diseases: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet Infect Dis.* (2017) 17:909–48. doi: 10.1016/S1473-3099(17)30276-1

2. Shah MP, Dahl RM, Parashar UD, Lopman BA. Annual changes in rotavirus hospitalization rates before and after rotavirus vaccine implementation in the United States. *PLoS ONE.* (2018) 13:e0191429. doi: 10.1371/journal.pone.0191429

3. GBD Diarrhoeal Diseases Collaborator. Estimates of the global, regional, national morbidity, mortality, and aetiologies of diarrhoea in 195 countries: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet Infect Dis.* (2018) 18:1211–28. doi: 10.1016/S1473-3099(18)30362-1

4. Shah MP, Hall AJ. Norovirus illnesses in children and adolescents. *Infect Dis Clin North Am.* (2018) 32:103–18. doi: 10.1016/j.idc.2017.11.004

5. Bruijning-Verhagen P, Nipshagen MD, de Graaf H, Bonten MJM. Rotavirus disease course among immunocompromised patients; 5-year observations from a tertiary care medical centre. *J Infect.* (2017) 75:448–54. doi: 10.1016/j.jinf.2017.08.006

6. Posovszky C, Buderus S, Classen M, Lawrenz B, Keller KM, Koletzko S. Acute infectious gastroenteritis in infancy and childhood. *Dtsch Arztebl Int.* (2020) 117:615–24. doi: 10.3238/arztebl.2020.0615

7. Koho T, Huhti L, Blazevic V, Nurminen K, Butcher SJ, Laurinmaki P, et al. Production and characterization of virus-like particles and the P domain protein of GII4 norovirus. *J Virol Methods.* (2012) 179:1–7. doi: 10.1016/j.jviromet.2011.05.009

8. Liu H, Rong P, Jia H, Yang J, Dong B, Dong Q, et al. A wash-free homogeneous colorimetric immunoassay method. *Theranostics.* (2016) 6:54–64. doi: 10.7150/thno.13159

9. Kim J, Kim HS, Kim HS, Kim JS, Song W, Lee KM, et al. Evaluation of an immunochromatographic assay for the rapid and simultaneous detection of rotavirus and adenovirus in stool samples. *Ann Lab Med.* (2014) 34:216–22. doi: 10.3343/alm.2014.34.3.216

10. Mousavi-Nasab SD, Sabahi F, Kaghazian H, Paryan M, Mirab Samiee S, Ghaderi M, et al. A real-time RT-PCR assay for genotyping of rotavirus. *Iran Biomed J.* (2020) 24:399–404. doi: 10.29252/ibj.24.6.394

11. Yu ZT, Guan H, Cheung MK, McHugh WM, Cornell TT, Shanley TP, et al. Rapid, automated, parallel quantitative immunoassays using highly integrated microfluidics and AlphaLISA. *Sci Rep.* (2015) 5:11339. doi: 10.1038/srep11339

12. Zhao J, Lv Q, Liu P, Guo L, Zhang L, Zheng Y, et al. AlphaLISA for detection of staphylococcal enterotoxin B free from interference by protein A. *Toxicon.* (2019) 165:62–8. doi: 10.1016/j.toxicon.2019.04.016

13. Liu J, Gratz J, Amour C, Kibiki G, Becker S, Janaki L, et al. A laboratory-developed TaqMan Array Card for simultaneous detection of 19 enteropathogens. *J Clin Microbiol.* (2013) 51:472–80. doi: 10.1128/JCM.02658-12

14. Bian Y, Liu C, She T, Wang M, Yan J, Wei D, et al. Development of a light-initiated chemiluminescent assay for the quantitation of sIgE against egg white allergens based on component-resolved diagnosis. *Anal Bioanal Chem.* (2018) 410:1501–10. doi: 10.1007/s00216-017-0791-y

15. Nguyen GT, Phan K, Teng I, Pu J, Watanabe T. A systematic review and meta-analysis of the prevalence of norovirus in cases of gastroenteritis in developing countries. *Medicine.* (2017) 96:e8139. doi: 10.1097/MD.0000000000008139

16. Wongboot W, Okada K, Chantaroj S, Kamjumphol W, Hamada S. Simultaneous detection and quantification of 19 diarrhea-related pathogens with a quantitative real-time PCR panel assay. *J Microbiol Methods.* (2018) 151:76–82. doi: 10.1016/j.mimet.2018.06.006

17. Ha C, Yoo IY, Yun SA, Chung YN, Huh HJ, Lee NY. Performance evaluation of Automated Fluorescent Immunoassay System ROTA and NORO for detection of rotavirus and norovirus: A comparative study of assay performance with RIDASCREEN((R)) Rotavirus and Norovirus. *J Clin Lab Anal.* (2021) 35:e23585. doi: 10.1002/jcla.23585

18. Wang Y, Zhang J, Liu P. Clinical and molecular epidemiologic trends reveal the important role of rotavirus in adult infectious gastroenteritis, in Shanghai, China. *Infect Genet Evol.* (2017) 47:143–54. doi: 10.1016/j.meegid.2016.11.018

19. Liu TC, Huang H, Dong ZN, He A, Li M, Wu YS, et al. Development of an amplified luminescent proximity homogeneous assay for quantitative determination of hepatitis B surface antigen in human serum. *Clin Chim Acta.* (2013) 426:139–44. doi: 10.1016/j.cca.2013.09.013

20. Hou C, Zhao L, Geng F, Wang D, Guo LH. Donor/acceptor nanoparticle pair-based singlet oxygen channeling homogenous chemiluminescence immunoassay for quantitative determination of bisphenol A. *Anal Bioanal Chem.* (2016) 408:8795–804. doi: 10.1007/s00216-016-9584-y

21. Li P, Chen Z, Liu B, Li K, Wang H, Lin L, et al. Establishment of a novel homogeneous nanoparticle-based assay for sensitive procalcitonin detection of ultra low-volume serum samples. *Int J Nanomedicine.* (2018) 13:5395–404. doi: 10.2147/IJN.S173776

22. Chen D, Wang D, Wang C, Wei F, Zhao H, Lin X, et al. Application of an AlphaLISA method for rapid sensitive detection of African swine fever virus in porcine serum. *Appl Microbiol Biotechnol.* (2021) 105:4751–9. doi: 10.1007/s00253-021-11339-2

23. Waller H, Chatterji U, Gallay P, Parkinson T, Targett-Adams P. The use of AlphaLISA technology to detect interaction between hepatitis C virus-encoded NS5A and cyclophilin A. *J Virol Methods.* (2010) 165:202–10. doi: 10.1016/j.jviromet.2010.01.020

24. Gorshkov K, Chen CZ, de la Torre JC, Martinez-Sobrido L, Moran T, Zheng W. Development of a high-throughput homogeneous AlphaLISA drug screening assay for the detection of SARS-CoV-2 Nucleocapsid. *bioRxiv.* (2020). doi: 10.1021/acsptsci.0c00122

25. Zou LP, Liu TC, Lin GF, Dong ZN, Hou JY Li M, et al. AlphaLISA for the determination of median levels of the free beta subunit of human chorionic

gonadotropin in the serum of pregnant women. *J Immunoassay Immunochem.* (2013) 34:134–48. doi: 10.1080/15321819.2012.690358

26. Kimpston-Burkgren K, Mora-Diaz JC, Roby P, Bjustrom-Kraft J, Main R, Bosse R, et al. Characterization of the humoral immune response to porcine epidemic diarrhea virus infection under experimental and field conditions using an AlphaLISA platform. *Pathogens*. (2020) 9:233. doi: 10.3390/pathogens9030233

27. Zhang L, Lv Q, Zheng Y, Chen X, Kong D, Huang W, et al. A rapid and accurate method for screening T-2 toxin in food and feed using competitive AlphaLISA. *FEMS Microbiol Lett*. (2021) 368:fnab029. doi: 10.1093/femsle/fnab029

28. Corcoran MS, van Well GT, van Loo IH. Diagnosis of viral gastroenteritis in children: interpretation of real-time PCR results and relation to clinical symptoms. *Eur J Clin Microbiol Infect Dis*. (2014) 33:1663–73. doi: 10.1007/s10096-014-2135-6

29. Kaplon J, Thery L, Bidalot M, Grangier N, Frappier J, Aho Glele LS, et al. Diagnostic accuracy of four commercial triplex immunochromatographic tests for rapid detection of rotavirus, adenovirus, and norovirus in human stool samples. *J Clin Microbiol*. (2020) 59:e01749-20. doi: 10.1128/JCM.01749-20

frontiers | Frontiers in Medicine

TYPE Original Research
PUBLISHED 11 August 2022
DOI 10.3389/fmed.2022.940332

OPEN ACCESS

EDITED BY
Md Robiul Karim,
Bangabandhu Sheikh Mujibur Rahman Agricultural University, Bangladesh

REVIEWED BY
Milad Badri,
Qazvin University of Medical Sciences, Iran
Mrigya Babuta,
Beth Israel Deaconess Medical Center and Harvard Medical School, United States

*CORRESPONDENCE
Majid Pirestani
pirestani@modares.ac.ir

SPECIALTY SECTION
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

RECEIVED 10 May 2022
ACCEPTED 27 July 2022
PUBLISHED 11 August 2022

CITATION
Norouzi M, Pirestani M, Arefian E, Dalimi A, Sadraei J and Mirjalali H (2022) Exosomes secreted by *Blastocystis* subtypes affect the expression of proinflammatory and anti-inflammatory cytokines (TNFα, IL-6, IL-10, IL-4). *Front. Med.* 9:940332. doi: 10.3389/fmed.2022.940332

Exosomes secreted by *Blastocystis* subtypes affect the expression of proinflammatory and anti-inflammatory cytokines (TNFα, IL-6, IL-10, IL-4)

Mojtaba Norouzi[1], Majid Pirestani[1]*, Ehsan Arefian[2], Abdolhossein Dalimi[1], Javid Sadraei[1] and Hamed Mirjalali[3]

[1]Department of Parasitology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran, [2]Department of Microbiology, School of Biology, College of Science, University of Tehran, Tehran, Iran, [3]Foodborne and Waterborne Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Background: *Blastocystis* sp. is a common intestinal parasite, possibly responsible for diarrhea, vomiting and nausea, abdominal pain, and irritable bowel syndrome. However, many studies focused on this issue due to the uncertainty of its pathogenic potential. The extracellular vesicles (EVs) are significant mediators for cellular communication, carrying biological molecules such as proteins, lipids, and nucleic acids. Compared with other parasites, little is known about the *Blastocystis* EVs. Hence the present investigation was done.

Methods: The *Blastocystis* parasites were cultured in the DMEM medium, and a 550–585 bp fragment was amplified using PCR, and sequencing was done. A commercial kit was used for exosome extraction and dynamic light scattering (DLS), flow cytometry (CD63, CD81 markers), and electron microscopy tests to determine their morphology. The human leukemia monocytic cell line (THP-1) was exposed to *Blastocystis* EVs. Next, the expression of proinflammatory and anti-inflammatory cytokines, including IL-4, IL-6, IL-10, and tumor necrosis factor-alpha (TNF-α), were measured using quantitative PCR.

Results: Exosomes were extracted from ST1-3 *Blastocystis* sp. According to the DLS assay, the size of the exosomes was in the range of 30–100 nm. Electron microscopy images and CD63 and CD81 markers also confirmed the exosome's size, structure, and morphology. According to real-time PCR results, ST1-derived exosomes caused IL-6 and TNF-α upregulation and IL-10 and IL-4 downregulation, ST2- and ST3-derived exosomes downregulated IL-10, and ST3-derived exosomes caused IL-6 upregulation. There is a statistically significant difference ($P \leq 0.05$).

Conclusion: To our knowledge, this is the first report of the release of exosome-like vesicles by the human parasite, *Blastocystis*, and the provided information demonstrates the role of this parasite, particularly ST1 on proinflammatory and anti-inflammatory cytokines and navigating the host response.

KEYWORDS

exosomes, *Blastocystis* subtypes, proinflammatory, anti-inflammatory, cytokines

Introduction

Parasitic diseases contribute to high morbidity and mortality yearly, particularly in developing countries (1). The parasitic agents employ many molecular mechanisms to communicate and manipulate the host responses (2). The extracellular vesicles (EVs) have been recognized as novel mediators in maintaining intestinal homeostasis, principally in the intestinal mucosa (3). These nano-molecules are also implicated in coordinating the growth and development, horizontal gene transfer, and host-pathogen communications, particularly at the early stages of the infection. Several mechanisms exist for EV uptake by host cells (4, 5). Phagocytosis and macropinocytosis are primarily involved in EV uptake, surrounded by the plasma membrane (6). Other entry routes are receptor-mediated contact, fusion with the target cell plasma membrane, and delivery of the soluble cargo to the cells. Reportedly, the information carried by EVs isis involved in disease development (7). EVs have been known to possess clinical applications, both as diagnostic biomarkers and therapeutic agents (8).

There are different types of EVs based on size and biogenesis. Exosomes (30–100 nm) are composed of late endosomes containing vesicles that combine with the plasma membrane, (9) and are released into the extracellular environment, microvesicles (100–1,000 nm) are directly germinated from the plasma membrane and are produced by outward budding of the plasma membrane and apoptotic bodies (1,000–5,000 nm) result from the interaction of myosin-actin during programmed cell death or apoptosis and contain cell contents such as organelles (10). Specific proteins named calpins with cysteine-dependent calcium protease activity, found in most eukaryotes and some prokaryotes, are involved in the releasing processes of EVs (11). In parasitic agents, the EVs may play a critical role in modulating the host's immune responses (12, 13). The following proteins are consistently found within exosomes: heat shock proteins (HSP60, HSP70, and HSP90), nucleic acids (DNA, RNA, microRNA), tetraspanins (CD81, CD63, and CD37), anxins (I, II, V and VI), cytoskeletal proteins (actin and tubulin), metabolic enzymes, proteins involved in translation (elongation factors 1 and 2) and signaling proteins (14, 15). These biological macromolecules are particularly interested in the diagnosis of infectious disease pathology (16).

The extracellular protozoan parasite, *Blastocystis* sp., is a gastrointestinal inhabitant in animals and humans, being assigned to cause abdominal pain, diarrhea, vomiting, and nausea. It has also been speculated to be involved in the initiation and or progression of irritable bowel syndrome (IBS) (17, 18), inflammatory bowel disease (IBD), and colorectal cancer (19). The phylogenetic analysis has placed the *Blastocystis* within the Stramenopiles group, along with brown algae (20). Among 28 identified subtypes (STs) of *Blastocystis*, nine (ST1-ST9) have been found in humans (21). Based on the published literature, subtypes 1–3 have been linked to chronic urticaria and skin disorders (1, 22, 23). Moreover, *Blastocystis* can activate cytokine production and immune responses *in vitro* (24). Cysteine protease enzymes in *Blastocystis* (especially ST7) can induce myosin light chain phosphorylation, ZO1 protein degradation, and F-actin reorganization in the Caco-2 cell line (25, 26). Compared with other parasites, little is known about the characterization, function, and host-parasite interaction of the *Blastocystis* EVs (27). Therefore, the present experiment investigated the effects of the *Blastocystis* exosomes on the expression of proinflammatory and anti-inflammatory cytokines such as IL-6, TNFα, IL-4, and IL-10 using molecular techniques.

Abbreviations: TNF-α, tumor necrosis factor-alpha; DMEM, Dulbecco's Modified Eagle Medium; EVs, Extracellular vehicles; GI, gastrointestinal; IBS, Irritable Bowel Syndrome; IBD, Inflammatory Bowel Disease; BSA, Bovine Serum Albumin; SEM, Scanning Electron Microscope; TEM, Transmission Electron Microscopy; PMA, Phorbol Myristate Acetate; DLS, Dynamic Light Scattering.

Materials and methods

Parasite culture

Positive *Blastocystis* samples were cultured in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and Penicillin-Streptomycin (1,000-unit penicillin and 4 mg/ml streptomycin) (Figure 1).

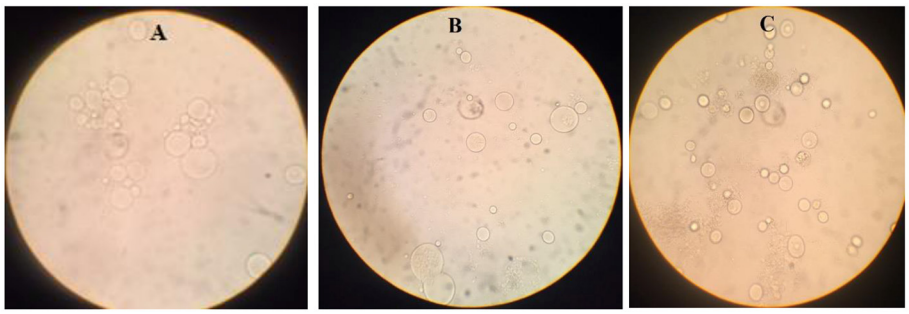

FIGURE 1
Blastocystis sp. in the medium (DMEM) with FBS and penicillin-streptomycin [**(A)**: Subtype 3, **(B)**: Subtype 2, **(C)**: Subtype 1].

DNA extraction, polymerase chain reaction, and sequencing

Upon propagation of the parasites in the culture medium, DNA was extracted using DNA Extraction Kit (SINACLON), based on the manufacturer's protocol, and a ~600 bp fragment of the *Blastocystis* small subunit ribosomal DNA (SSU rDNA) gene was amplified using PCR method, according to the previous studies (Figure 2) (28). The amplification was done using a forward primer, RD5 (ATCTGGTTGATCCTGCCAGT) (29), and a reverse primer, BhRDr (GAGCTTTTTAACTGCAACAACG) (30).

The PCR amplicons were run on a 1% gel electrophoresis and visualized using ultraviolet (UV) illumination in a Gel-Doc apparatus. Sequencing was performed by the Sanger method. All obtained sequences were edited by SEQUNCHERTM software (ver. 5.4.5), and a comparison with other sequences deposited in the GenBank was performed using the basic local alignment tool (BLAST), available at http://blast.ncbi.nlm.nih.gov. Next, subtypes 1, 2, and 3 of *Blastocystis* sp. were selected for exosome extraction and further evaluation.

Exosome extraction

Initially, an FBS-free DMEM medium was used to culture the parasites for 48 h before extraction. A commercial kit, Exocib (Cib Biotech Co., IRI), was used for exosome extraction, and the extraction steps were done based on the manufacturer's guidelines. The purified exosomes were stored at 4°C for a few days or kept at −20°C or −80°C for a long time.

Quantification of exosome concentration

For this purpose, a kit-based Bradford test (Diba NoAvaran Azma Company, DNAbiotech) was used. The standard bovine serum albumin (BSA) was diluted with 1 mg/ml storage solution (100 μl BSA + 900 μl PBS or distilled water) and prepared based on the dilution kit protocol. A standard graph was plotted, and the trendline, the line equation, and the regression coefficient were determined on the graph. Finally, 20 μl of each sample was mixed with 180 μl of the reagent, and the optical density (absorbance) was read at 595 nm after 10 min.

Exosome characterization

Dynamic light scattering

This procedure was done to determine the extracted exosomes' size distribution. For this aim, 50 μl of exosome sample for each subtype of interest was diluted in 950 μl of PBS. Then, the size distribution of the exosomes was read by Zetasizer Nano (ZS (Malvern Instruments, Southborough, UK) at 25 ° C with a refractive index of 1.38 and absorption of 0.01.

Evaluation of exosome surface markers by flow cytometry technique

The exosome surface markers, CD63 and CD81, were evaluated using the flow cytometry technique. Initially, the exosomes were connected to sulfate aldehyde sulfate beads (size: 4 μm) to improve their size for the reader device. Briefly, 30 μg of the exosomes from each subtype was incubated with 90 μl of bead for 95 min at ambient temperature. Subsequently, the volume of the samples was increased to 1 ml with PBS and placed on a test tube rotator at 4°C for 16 h. Then the attachment process was terminated by adding 110 μl of 100 mM glycine solution and incubating for 30 min at room temperature. The exosome-coated beads were triple washed with PBS containing 0.5% BSA. Then, the exosome-bead complex was incubated with anti-CD63 and anti-CD81 antibodies separately for each group for about 1 h at 4°C. In the final step, the expression of the mentioned markers was examined by the flow cytometry method.

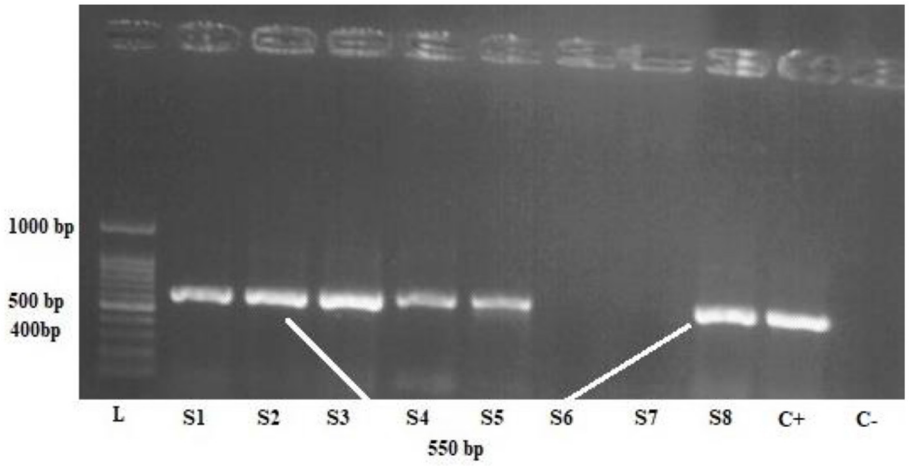

FIGURE 2
One percent agarose gel electrophoresis of the polymerase chain reaction product of *Blastocystis* samples, L: 100-bpDNA ladder, C + positive control, C- negative control, S1,2,3,4,5,8 positive samples.

Evaluation the size, structure, and morphology of exosome

Scanning electron microscopy

The morphology and size of the exosomes were also monitored using SEM imaging. First, the samples were fixed using pre-cooled 2.5 % glutaraldehyde solution (Sigma–Aldrich, Germany), placed on the specimen stub, and snap-frozen in a freeze-dryer (Model 5006; Dena Vacuum Industry Co., Ltd). Then, the specimens were sputter-coated with gold-palladium and imaged by the SEM system.

Transmission electron microscopy

Purified exosomes were diluted with an equal volume of 4% paraformaldehyde at 4°C for 30 min, then carefully placed on a carbon-coated 300-mesh copper grid for 20 min, followed by a fixation step using 1% glutaraldehyde for 5 min. The mesh was contrasted with 2% uranyl acetate, washed twice, and the morphology of the isolated exosomes was observed. Exosome images retrieved through TEM (TEM, LEO 906, Zeiss, Germany) were analyzed by ImageJ software.

Expression of inflammatory and proinflammatory cytokines in the presence of exosomes

Cell culture

The Roswell Park Memorial Institute (RPMI 1,640) medium supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% L-glutamine was used to culture the human leukemia monocytic cell line (THP-1) (Figure 3). The cells were enumerated with trypan blue (0.4%), and 400 × 10^3 cells were seeded in each well of 12-well plates and subsequently treated by 50 ng phorbol myristate acetate (PMA) overnight, then washed three times. The cells were then rested for a day.

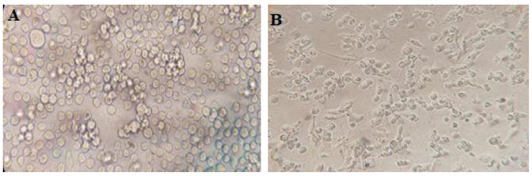

FIGURE 3
(A) THP-1 cell in RPMI 1,640 medium and **(B)** THP-1 macrophages in RPMI 1,640 medium.

Exposure of THP-1 cells to exosomes

Prepared THP-1 cells were exposed to 10 μg of exosomes from each subtype, being incubated for 24 h. Next, the cells were trypsinized and collected in a microtube for the next steps.

RNA extraction

RNA extraction was performed manually according to the following protocol: First, 1 ml of Trizol (Thermo Scientific) was added to the collected samples and a pipette, then 250 μl of chloroform was added and mixed. The next step was incubated at room temperature for 15 min, then centrifuged at 15,000 rpm at 4° C for 15 min. In the next step, the supernatant was removed and transferred to a new microtube, then 600–800 μl of cold

TABLE 1 List of primers were used in real-time PCR.

No.	Gene	Sequence		Ref
1	IL4	5′-ACAGCCTCACAGAGCAGAAGACT-3′	Forward	(31)
		5′-TGTGTTCTTGGAGGCAGCAA-3′	Reverse	(31)
2	IL-10	5′-GCAGTGGAGCAGGTGAAGAG-3′	Forward	(32)
		5′-CGGAGAGAGGTACAAACGAGG-3′	Reverse	(32)
3	IL-6	5′-AGTTGCCTTCTTGGGACTGA-3′	Forward	(31)
		5′-TCCACGATTTCCCAGAGAAC-3′	Reverse	(31)
4	TNF-α	5′-GAACTGGCAGAAGAGGCACT-3′	Forward	(32)
		5′-AGGGTCTGGGCCATAGAACT-3′	Reverse	(32)
5	β-actin	5′-GCCATGTACGTAGCCATCCA-3′	Forward	(33)
		5′-ACGCACGATTTCCCTCTCAG-3′	Reverse	(33)

isopropanol was added to it and mixed together. The samples were kept at −20 °C for 24 h. They were then centrifuged at 13,000 rpm at 4 °C for 45 min. The supernatant was then removed, and 1 ml of 70% cold ethanol was added to the RNA precipitate (washing). The samples were centrifuged at 20,000 rpm at 4°C for 20 min. Remove the supernatant and allow the precipitate to dry for about 10 min (drying). Then 20 μl of sterile distilled water was added to the precipitate and gently pipetted to dissolve the RNA precipitate in water. The extracted RNA was stored at −70°C. The quality and concentration of RNA were evaluated with a nanodrop spectrophotometers (ND2000, Thermo Scientific). The OD 260/280 nm ratio was reported to be about 2.00.

CDNA synthesis and real-time PCR

For cDNA synthesis, total RNA was reverse-transcribed using M-MLV reverse transcriptase according to the manufacturer's instructions (RT-ROSET Kit). Real-time PCR was performed in triplicate using 50 ng of cDNA with RealQ Plus 2x Master Mix Green reagent (Ampliqon) in a Rotor Gene-Q thermal cycler (Qiagen). Primer sequences are shown in Table 1. Relative gene expression values were normalized to β-actin and calculated using the comparative CT method ($2^{-\Delta\Delta CT}$). The values are presented as mean n-fold differences compared to the control ($P \leq 0.05$ was reported to be significant).

Result

Subtyping of the *Blastocystis sp.*

The *Blastocystis* parasites were cultured in a DMEM medium enriched with 10% FBS and penicillin-streptomycin antibiotics, and a molecular investigation followed by sequencing of the propagated parasites revealed ST1-ST3. The sequences have been deposited in GenBank under accession numbers OL457221-OL457257. Multiple sequence alignments were performed using the ClustalW, and phylogenetic analyses using the maximum-likelihood (ML) method were carried out using the MEGA7 using all subtypes of *Blastocystis*. The tree was constructed (500 replicates) using the 18S rRNA gene sequence of Proteromonas laceratae (U37108) as an outgroup (Figure 4).

Determination of the concentration of extracted exosomes

Based on the Bradford assay, the concentrations of the exosomes for subtypes 1, 2, and 3 included 1.9 μg/μl (ST1), 2.3 μg/μl (ST2), and 2.2 μg/μl (ST3).

Confirmatory tests for exosome extraction

DLS test was performed to determine the size of the exosomes. The peak of EVs population was obtained in the range of exosomes, and the intensity was 68.2% (Figure 5). Furthermore, the electron microscopy studies confirmed the homogeneity of the population with a size of 30–100 nm and their spherical morphology (Figures 6, 7). Also, due to existing limitations, we were not able to measure exosomes in the range of 100–1,000 nm. The flow cytometry results showed the expression of CD63 (94.33%) and CD81 (97.71%) markers on the surface of exosomes (Figure 8).

Expression of inflammatory and proinflammatory cytokines by THP-1 cells exposed to the exosomes

Next, the exosomes were exposed to THP-1 cells for 24 h, then cells were trypsinized, and RNA extraction, cDNA synthesis, and real-time PCR experiments were performed.

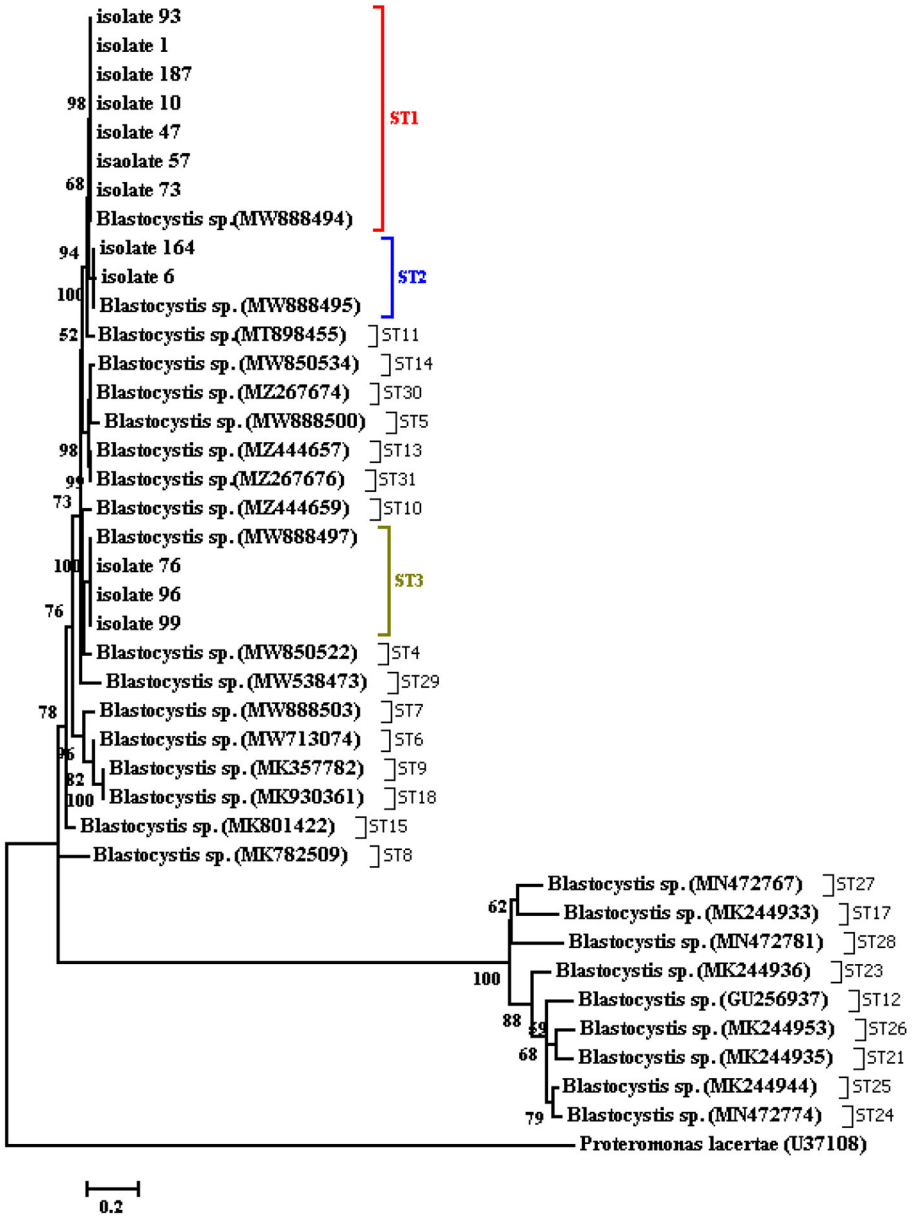

FIGURE 4
Phylogenetic tree of the SSU-rDNA gene sequences of *Blastocystis* isolates constructed by the neighbor-joining method using Mega4 software.

Analysis of real-time PCR output and fold change calculation showed (Table 2) that in ST1, the expression of IL-6 and TNF-α was upregulated, compared to the control group. Also, ST1 showed a decrease in IL-4 and IL-10 expression, while IL-4 expression was not changed in ST2- and ST3-exposed THP-1 cells (Figure 9). It is noteworthy that IL-10 showed a decrease in expression in all three subtypes compared to the control group Notably, The expression of TNF-α in exosome-exposed cells in subtype 1 was upregulated compared to the control (LPS).

Discussion

Blastocystis is an extracellular organism in the gut of animals and humans, which can suppress the inducible nitric oxide (iNOS) production and cleave the immunoglobulins, evading the host's immune system responses (34). This compromised molecular milieu also paves the way for other co-infections to infect the intestinal epithelium and cause disease (35). During the last decades, the molecular basis of the host-parasite cross-talk has been more elucidated, highlighting to be mediated by the

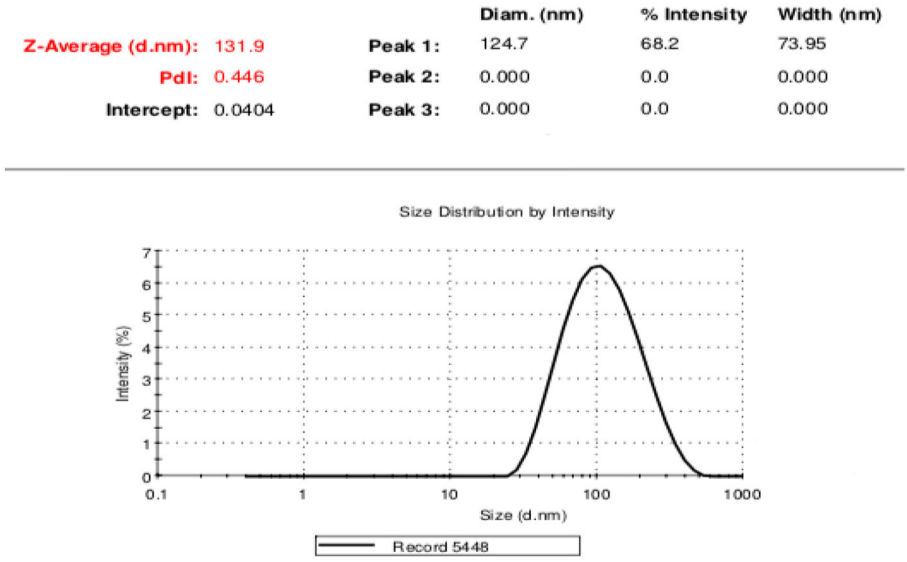

FIGURE 5
Extracellular vesicle size distribution using DLS techniq.

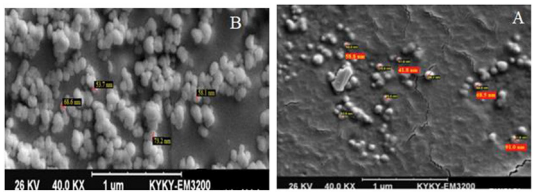

FIGURE 6
SEM electron microscope image of exosome specimens of *Blastocystis* subtypes [**(A)**: Exosomes without conjugation and **(B)**: with sulfate aldehyde sulfate beads].

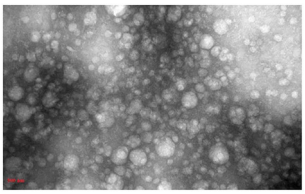

FIGURE 7
TEM electron microscope image of exosome specimens of *Blastocystis* subtypes.

EV nano-molecules (~30–5,000 nm) as tiny vehicles for cellular communication that may carry proteins, lipids, nucleic acids, and metabolites from their cellular origin, essential for genetic exchange, biomarker identification, and the diagnosis of diseases (36, 37).

Based on the *in vitro* experiments, *Blastocystis* parasites can elicit cytokine production and immune responses (38). Despite the availability of some *Blastocystis* subtype's genomes, our knowledge is still in its infancy on the biological and pathological mechanisms of the parasite (39). Curiously, membrane proteins and the integrity of the epithelial barrier could be substantially disrupted by the *Blastocystis* ST7 *via* degradation of tight proteins (3). So far, few studies have characterized EVs in *Blastocystis*, and the early evidence was provided by Tan through the TEM experiment (35). According to recent studies, *Blastocystis*-derived EVs were identified in ST7 B and H isolates, with cup-shaped morphology and an average size of 50 to 240 nm consistent with EVs derived from other parasites (40).

In recent years, a great deal of interest has been focused on the epidemiology, phylogeny, and cell biology of *Blastocystis*; only a few studies have addressed the parasite virulence and specific host responses (41). Based on the published literature, gastrointestinal symptoms similar to IBS, including diarrhea, abdominal pain, constipation, nausea, inflammation, and edema, have been reported in patients infected with *Blastocystis* (42). On the other hand, the parasite has been recognized mainly in asymptomatic individuals, thus possibly being non-invasive (43). Edema and infiltration of the inflammatory cells into the lamina propria have been reported in the cecum and colon of infected mice (44). An experiment showed that *Blastocystis* induces IL-8 production in colon T84 epithelial cells in a time-dependent manner, which provokes the inflammatory cells to invade the intestinal mucosa, resulting in tissue damage and gastrointestinal disorders (45).

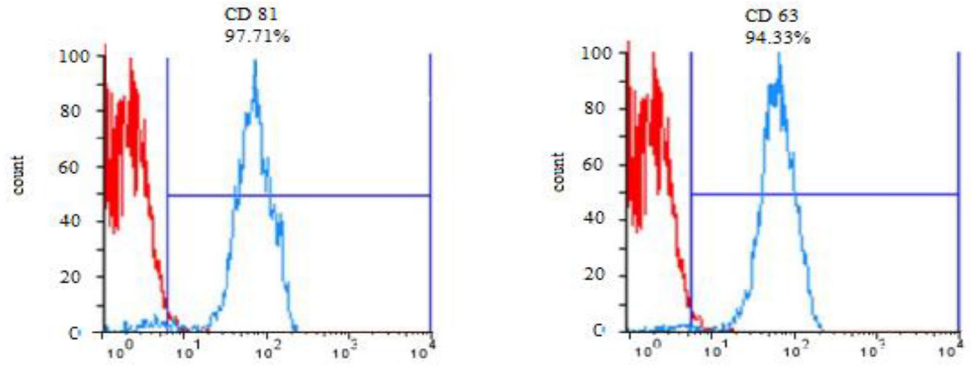

FIGURE 8
Expression of CD 63 and CD 81 surface markers in exosomes of *Blastocystis* subtypes.

TABLE 2 Results of real-time PCR data analysis.

Exosome	Cytokine	Expression	Fold change	*P*-value
Subtype 1	IL-4	Down Regulate	−67.323	<0.0001
	IL-10	Down Regulate	−53.259	<0.0001
	IL-6	UP Regulate	87.620	<0.0001
	TNFα	Not different	36.422	0.004
Subtype 2	IL-4	Not different	−19.849	0.999
	IL-10	Down Regulate	−78.385	<0.0001
	IL-6	Not different	35.677	0.580
	TNFα	Not different	22.576	0.994
Subtype 3	IL-4	Not different	−19.578	0.908
	IL-10	Down Regulate	−68.967	<0.0001
	IL-6	Down Regulate	43.188	0.002
	TNFα	Not different	23.287	0.998

The values are presented as mean n-fold differences compared to the control (P-value $\leq$ 0.05 was reported to be significant).

Previously, no investigation was done to evaluate the association between IL-6 and TNF-α gene polymorphisms in susceptibility to diseases such as IBS (46). Here, we evaluated such an association and revealed a significant association between IL-6 gene expression in THP-1 cells exposed to ST1 exosomes. This may suggest that the presence of a parasite such as *Blastocystis* would play a substantial role in IBS by promoting the clinical outcomes. Another major finding in the current study was that no significant difference was observed regarding TNF-α expression in comparison with the control group, which is inconsistent with Iguchi et al. study, demonstrating significant upregulation of IFN-γ, IL-12, and TNF-α cytokines in the cecal mucosa (47). Downregulation of IFN-γ and TNF-α, along with the upregulation of IL-6 and IL-8, has been observed in colorectal cancer (48). This evidence indicates that the *Blastocystis* antigen (Blasto-Ag), an example of the extracellular allergen, has stimulated the humoral responses, leading to inflammatory reactions and cell propagation to combat the infection (49). Another study by Yakoob et al. demonstrated significantly lower IL-10 levels and colonic eosinophilic infiltration associated with IL-8 in the *Blastocystis* ST1 compared to ST3 and control (32). In the current study, we found a reduction in the expression of IL-10 in ST1, ST2 and ST3. It has been recognized that IL-4 and IL-13 cytokines mediate the goblet cell hyperplasia during the gut infection, which the latter plays a significant protective role against the infection (50). Our results demonstrated a decrease in IL-4 and IL-10 expression and an increase in IL-6 and TNF-α expression in *Blastocystis* ST1. Reportedly, IL-10 is essential in regulating inflammatory responses, as it reduces the production of chemotactic factors such as IL-8 (51). Altogether, the increased mucin layer and fluid secretion, goblet cell hyperplasia, and enhanced intestinal propulsive activity result in the expulsion of noxious agents from the gut lumen (52).

Finally, the results presented here highlighted the significance of the *Blastocystis* ST1 exosomes on the expression of IL-6, IL-10, IL-4 and TNF-α cytokines,

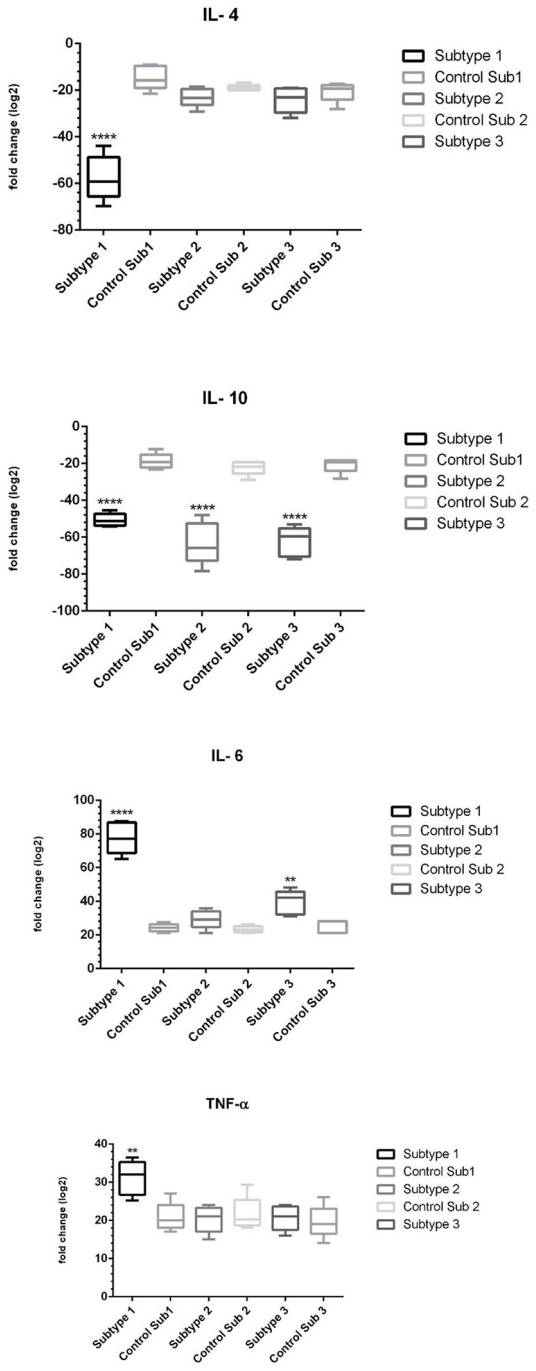

FIGURE 9
The expression of proinflammatory and anti-inflammatory cytokines in the presence of exosomes of subtypes 1, 2 and 3 of *Blastocystis* (The sign (*) in each subtype indicates which cytokine was statistically significant compared to control) (**$p < 0.01$; ****$p < 0.0001$).

strengthening the hypothesis that the *Blastocystis* parasite is a potent contributor to the inflammatory processes. To better understand the interaction of EVs of *Blastocystis*, other inflammatory cytokines such as MCP-1 and IL1B should be investigated. However, the actual pathogenicity of *Blastocystis* and its association with gastrointestinal symptoms is yet to be determined through extensive research, possibly in the field of EVs, their biogenesis, uptake, and cellular communication.

Conclusion

To our knowledge, this is the first report of the release of EVs by the human parasite *Blastocystis*, and our data demonstrate the role of this parasite, particularly ST1, on proinflammatory and anti-inflammatory cytokines and navigating the host responses. Further studies on the arrangement and function of these biologically-active vesicles could assist us in developing unprecedented therapeutic strategies, opening new doors toward the role of the *Blastocystis* parasite in gastrointestinal diseases.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Tarbiat Modares University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

MP and MN designed the study. EA and MP contributed to the methodology. EA and HM analyzed the data. AD, JS, and MN reviewed and edited the manuscript. EA was responsible for advising on controversial issues. MP is responsible for the overall content as a corresponding author. All authors have read and approved the final version of the manuscript.

Acknowledgments

This study is result of Ph.D. thesis that has been supported by Vice-Chancellor for Research of Tarbiat Modares University.

We are very grateful for the help and assistance of Mr. Alireza Olyaiee.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Mahmoud MS, Saleh WA. Secretory and humoral antibody responses to Blastocystis hominis in symptomatic and asymptomatic human infections. *J Egypt Soc Parasitol.* (2003) 33:13–30.

2. Liu Y, Rhoads JM. Communication between B-cells and microbiota for the maintenance of intestinal homeostasis. *Antibodies.* (2013) 2:535. doi: 10.3390/antib2040535

3. Turner JR. Intestinal mucosal barrier function in health and disease. *Nat Rev Immunol.* (2009) 9:799–809. doi: 10.1038/nri2653

4. Buzas EI, Gyorgy B, Nagy G, Falus A, Gay S. Emerging role of extracellular vesicles in inflammatory diseases. *Nat Rev Rheumatol.* (2014) 10:356–64. doi: 10.1038/nrrheum.2014.19

5. Evans-Osses I, Mojoli A, Monguio-Tortajada M, Marcilla A, Aran V, Amorim M, et al. Microvesicles released from Giardia intestinalis disturb host-pathogen response *in vitro*. *Eur J Cell Biol.* (2017) 96:131–42. doi: 10.1016/j.ejcb.2017.01.005

6. Olmos-Ortiz LM, Barajas-Mendiola MA, Barrios-Rodiles M, Castellano LE, Arias-Negrete S, Avila EE, et al. Trichomonas vaginalis exosome-like vesicles modify the cytokine profile and reduce inflammation in parasite-infected mice. *Parasite Immunol.* (2017) 39:e12426. doi: 10.1111/pim.12426

7. Goll R, Granlund AV. Intestinal barrier homeostasis in inflammatory bowel disease, Scand. *J Gastroenterol.* (2015) 50:3–12. doi: 10.3109/00365521.2014.971425

8. Babatunde KA, Mbagwu S, Hernandez-Castaneda MA, Adapa SR, Walch M, et al. Malaria infected red blood cells release small regulatory RNAs through extracellular vesicles. *Sci Rep*. (2018) 8:884. doi: 10.1038/s41598-018-19149-9

9. Cheng IS, Sealy BC, Tiberti N, Combes V. Extracellular vesicles, from pathogenesis to biomarkers: the case for cerebral malaria. *Vessel Plus*. (2020) 2020:17. doi: 10.20517/2574-1209.2020.08

10. Shirley DAT, Farr L, Watanabe K, Moonah S. A review of the global burden, new diagnostics, and current therapeutics for amebiasis. *Open Forum Infect Dis*. (2018) 5:ofy161. doi: 10.1093/ofid/ofy161

11. Akers JC, Gonda D, Kim R, Carter BS, Chen CC. Biogenesis of extracellular vesicles (EV): exosomes, microvesicles, retrovirus-like vesicles, and apoptotic bodies. *J Neuro-Oncol.* (2013) 113:1–11. doi: 10.1007/s11060-013-1084-8

12. Abels ER, Breakefield XO. Introduction to extracellular vesicles: biogenesis, RNA Cargo selection, content, release, and uptake. *Cell Mol Neurobiol.* (2016) 36:301–12. doi: 10.1007/s10571-016-0366-z

13. Bebawy M, Roseblade A, Luk F, Rawling T, Ung A, Grau GER. Cell-derived microparticles: new targets in the therapeutic management of disease. *J Pharm Pharm Sci.* (2013) 16:238–53. doi: 10.18433/J3989X

14. SchoreyJS. Bhatnagar S. Exosome function: from tumor immunology to pathogen biology. *Traffic*. (2008) 9:871–81. doi: 10.1111/j.1600-0854.2008.00734.x

15. Silverman JM, Clos J, de Oliveira CC, Shirvani O, Fang Y, Wang C, et al. An exosome-based secretion pathway is responsible for protein export from Leishmania and communication with macrophages. *J Cell Sci.* (2010) 123:842–52. doi: 10.1242/jcs.056465

16. Atayde VD, Aslan H, Townsend S, Hassani K, Kamhawi S, Olivier M. Exosome secretion by the parasitic protozoan leishmania within the sand fly midgut. *Cell Rep*. (2015) 13:957–67. doi: 10.1016/j.celrep.2015.09.058

17. Battistelli M, Falcieri E. Apoptotic bodies: particular extracellular vesicles involved in intercellular communication. *Biology (Basel).* (2020) 9:21. doi: 10.3390/biology9010021

18. Barbosa CV, Barreto MM, Andrade RJ, Sodré F, d'Avila-Levy CM, Peralta JM, et al. Intestinal parasite infections in a rural community of Rio de Janeiro (Brazil): prevalence and genetic diversity of Blastocystis subtypes. *PLoS ONE.* (2018) 13:e0193860. doi: 10.1371/journal.pone.0193860

19. Hublin JS, Maloney JG, Santin M. Blastocystis in domesticated and wild mammals and birds. *Res Vet Sci.* (2021) 135:260–82. doi: 10.1016/j.rvsc.2020.09.031

20. Greige S, El Safadi D, Khaled S, Gantois N, Baydoun M, Chemaly M, et al. First report on the prevalence and subtype distribution of Blastocystis sp. in dairy cattle in Lebanon and assessment of zoonotic transmission. *Acta Trop.* (2019) 194:23–9. doi: 10.1016/j.actatropica.2019.02.013

21. Danišová O, Valenčáková A. First detection of Blastocystis sp. in pigs in Slovakia and in Europe. *Parasitol Int.* (2021) 81:102235. doi: 10.1016/j.parint.2020.102235

22. Dogruman-Al F, Kustimur S, Yoshikawa H, Tuncer C, Simsek Z, Tanyuksel M, et al. Blastocystis subtypes in irritable bowel syndrome and inflammatory bowel disease in Ankara, Turkey, Mem. *Inst Oswaldo Cruz.* (2009) 104:724–7. doi: 10.1590/S0074-02762009000500011

23. Kumarasamy V, Kuppusamy UR, Jayalakshmi P, Samudi C, Ragavan ND, Kumar S. Exacerbation of colon carcinogenesis by Blastocystis sp. *PLoS ONE.* (2017) 12:e0183097. doi: 10.1371/journal.pone.0183097

24. Bahrami F, Babaei E, Badirzadeh A, Riabi TR, Abdoli A. Blastocystis, urticaria, and skin disorders: review of the current evidences. *Eur J Clin Microbiol Infect Dis.* (2020) 39:1027–42. doi: 10.1007/s10096-019-03793-8

25. Wu B, Yin J, Texier C, Roussel M, Tan KS. Blastocystis legumain is localized on the cell surface, and specific inhibition of its activity implicates a prosurvival role for the enzyme. *J Biol Chem.* (2010) 285:1790–8. doi: 10.1074/jbc.M109.049064

26. Wawrzyniak I, Texier C, Poirier P, Viscogliosi E, Tan KSW, Delbac F, et al. Characterization of two cysteine proteases secreted by Blastocystis ST7, a human intestinal parasite. *Parasitol Int.* (2012) 61:437–42. doi: 10.1016/j.parint.2012.02.007

27. Nourrisson C, Wawrzyniak I, Cian A, Livrelli V, Viscogliosi E, Delbac F, et al. On Blastocystis secreted cysteine proteases: a legumain-activated cathepsin B increases paracellular permeability of intestinal Caco-2 cell monolayers. *Parasitology.* (2016) 143:1713–22. doi: 10.1017/S0031182016001396

28. Hoevers JD, Snowden KF. Analysis of the ITS region and partial ssu and lsu rRNA genes of Blastocystis and Proteromonas lacertae. *Parasitology.* (2005) 131:187–96. doi: 10.1017/S0031182005007596

29. Howe EHY, Singh M. Do Blastocystis hominis colony forms undergo programmed cell death? *Parasitol Res.* (2001) 87:362–7. doi: 10.1007/s004360000364

30. Scanlan PD, Stensvold CR, Cotter PD. Development and application of a Blastocystis subtype-specific PCR assay reveals that mixed-subtype infections are common in a healthy human population. *Appl Environ Microbiol.* (2015) 81:4071–6. doi: 10.1128/AEM.00520-15

31. Loots GG, Locksley RM, Blank spoor CM, Wang ZE, Miller W, Rubin EM, et al. Identification of a coordinate regulator of interleukins 4, 13, and 5 by cross-species sequence comparisons. *Science.* (2000) 288:136–40. doi: 10.1126/science.288.5463.136

32. Yakoob J, Abbas Z, Usman MW, Sultana A, Islam M, Awan S, et al. Cytokine changes in colonic mucosa associated with Blastocystis spp. subtypes 1 and 3 in diarrhoea-predominant irritable bowel syndrome. *Parasitology.* (2014) 141:957–69. doi: 10.1017/S003118201300173X

33. Knerr I, Beinder E, Rascher W. Syncytin, a novel human endogenous retroviral gene in human placenta: evidence for its dysregulation in preeclampsia and HELLP syndrome. *Am J Obstet Gynecol.* (2002) 186:210–3. doi: 10.1067/mob.2002.119636

34. Tan KS, Mirza H. *Blastocystis–Host Interactions: Insights from In Vitro Model Systems. In Blastocystis: Pathogen or Passenger?* Heidelberg: Springer, Berlin. (2012), pp. 51–63.

35. Tan KS. New insights on classification, identification, and clinical relevance of *Blastocystis spp*. *Clin Microbiol Rev.* (2008) 21:639–65. doi: 10.1128/CMR.00022-08

36. Allen AR, Skuce RA, Byrne AW. Bovine tuberculosis in Britain and Ireland–a perfect storm? The confluence of potential ecological and epidemiological impediments to controlling a chronic infectious disease. *Front Vet Sci.* (2018) 5:109. doi: 10.3389/fvets.2018.00109

37. Liangsupree T, Multia E, Riekkola ML. Modern isolation and separation techniques for extracellular vesicles. *J Chromatogr A.* (2021) 1636:461773. doi: 10.1016/j.chroma.2020.461773

38. Ullah M, Qiao Y, Concepcion W, Thakor AS. Stem cell-derived extracellular vesicles: role in oncogenic processes, bioengineering potential, and technical challenges. *Stem Cell Res Ther.* (2019) 10:1–0. doi: 10.1186/s13287-019-1468-6

39. Nievas YR, Lizarraga A, Salas N, Cóceres VM, de Miguel N. Extracellular vesicles released by anaerobic protozoan parasites: current situation. *Cell Microbiol.* (2020) 22:e13257. doi: 10.1111/cmi.13257

40. Kumarasamy V, Anbazhagan D, Subramaniyan V, Vellasamy S. Blastocystis sp, parasite associated with gastrointestinal disorders: an overview of its pathogenesis, immune modulation and therapeutic strategies. *Curr Pharm Des.* (2018) 24:3172–5. doi: 10.2174/1381612824666180807101536

41. Wu ZN, Mirza H, Teo JDW, Tan KSW. Strain-dependent induction of human enterocyte apoptosis by Blastocystis disrupts epithelial barrier and ZO-1 organization in a caspase 3-and 9-dependent manner. *Biomed Res Int.* (2014) 1–11. doi: 10.1155/2014/209163

42. Carrera-Bravo C, Koh EY, Tan KS. The roles of parasite-derived extracellular vesicles in disease and host-parasite communication. *Parasitol Int.* (2021) 83:102373. doi: 10.1016/j.parint.2021.102373

43. Moe KT, Singh M, Howe J, Ho LC, Tan SW, Chen XQ, et al. Experimental Blastocystis hominis infection in laboratory mice. *Parasitol Res.* (1997) 83:319–25. doi: 10.1007/s004360050256

44. Li J, Deng T, Li X, Cao G, Li X, Yan Y, et al. rat model to study Blastocytis subtype 1 infections. *Parasitol Res.* (2013) 112:3537–41. doi: 10.1007/s00436-013-3536-7

45. Puthia MK, Lu J, Tan KS. Blastocystis ratti contains cysteine proteases that mediate interleukin-8 response from human intestinal epithelial cells in an NF-κB-dependent manner. *Eukaryot Cell.* (2008) 7:435–43. doi: 10.1128/EC.00371-07

46. Jimenez-Gonzalez DE, Martinez-Flores WA, Reyes-Gordillo J, Ramirez-Miranda ME, Arroyo-Escalante S, Romero-Valdovinos M, et al. Blastocystis infection is associated with irritable bowel syndrome in a Mexican patient population. *Parasitol Res.* (2012) 110:1269–75. doi: 10.1007/s00436-011-2626-7

47. Iguchi A, Yoshikawa H, Yamada M, Kimata I, Arizono N. Expression of interferon gamma and proinflammatory cytokines in the cecal mucosa of rats experimentally infected with Blastocystis sp. strain RN94-9. *Parasitol Res.* (2009) 105:135–40. doi: 10.1007/s00436-009-1373-5

48. Chandramathi S, Suresh K, Kuppusamy UR. Solubilized antigen of Blastocystis hominis facilitates the growth of human colorectal cancer cells, HCT116. *Parasitol Res.* (2010) 106:941–5. doi: 10.1007/s00436-010-1764-7

49. Zhang J, Zhu S, Ma N, Johnston LJ, Wu C, Ma X. Metabolites of microbiota response to tryptophan and intestinal mucosal immunity: a therapeutic target to control intestinal inflammation. *Med Res Rev.* (2021) 41:1061–88. doi: 10.1002/med.21752

50. Khan WI, Collins SM. Immune-mediated alteration in gut physiology and its role in host defence in nematode infection. *Parasite Immunol.* (2004) 26:319–26. doi: 10.1111/j.0141-9838.2004.00715.x

51. Jinquan T, Larsen CG, Gesser B, Matsushima K, Thestrup-Pedersen K. Human IL-10 is a chemoattractant for CD8+ T lymphocytes and an inhibitor of IL-8-induced CD4+ T lymphocyte migration. *J Immunol.* (1993) 151:4545–51.

52. Camilleri M. Leaky gut: mechanisms, measurement and clinical implications in humans. *Gut.* (2019) 68:1516–26. doi: 10.1136/gutjnl-2019-318427

frontiers | Frontiers in Medicine

TYPE Original Research
PUBLISHED 12 January 2023
DOI 10.3389/fmed.2022.1033417

OPEN ACCESS

EDITED BY
Sam Donta,
Falmouth Hospital, United States

REVIEWED BY
Duolong Zhu,
Baylor College of Medicine, United States
M. Jahangir Alam,
University of Houston, United States

*CORRESPONDENCE
Lucy Hocking
✉ lhocking@randeurope.org

SPECIALTY SECTION
This article was submitted to Infectious Diseases: Pathogenesis and Therapy, a section of the journal Frontiers in Medicine

RECEIVED 31 August 2022
ACCEPTED 23 December 2022
PUBLISHED 12 January 2023

CITATION
Hocking L, Wilcox M, Petrosillo N, Griffin P, Steiner T, Attara G, Doré J, Cabling M, Stockwell S, Romanelli JR and Marjanovic S (2023) Improving care for patients with *Clostridioides difficile* infection: A clinical practice and healthcare systems perspective. *Front. Med.* 9:1033417.
doi: 10.3389/fmed.2022.1033417

Improving care for patients with *Clostridioides difficile* infection: A clinical practice and healthcare systems perspective

Lucy Hocking[1]*, Mark Wilcox[2,3], Nicola Petrosillo[4], Paul Griffin[5], Theodore Steiner[6], Gail Attara[7,8], Joel Doré[9], Mark Cabling[1], Stephanie Stockwell[1], Robert J. Romanelli[1] and Sonja Marjanovic[1]

[1]RAND Europe, Cambridge, United Kingdom, [2]School of Medicine, University of Leeds, Leeds, United Kingdom, [3]Leeds Teaching Hospitals NHS Trust, Leeds, United Kingdom, [4]Policlinico Universitario, Bio-Medico Campus University Hospital, Rome, Italy, [5]Mater Clinical Unit, Faculty of Medicine, The University of Queensland, Brisbane, QLD, Australia, [6]Division of Infectious Diseases, The University of British Columbia, Vancouver, BC, Canada, [7]Canadian Society of Intestinal Research, Vancouver, BC, Canada, [8]Gastrointestinal Society, Vancouver, BC, Canada, [9]INRAE, MetaGenoPolis, AgroParisTech, Micalis Institute, Université Paris-Saclay, Jouy-en-Josas, France

Introduction: Arriving at a *C. difficile* infection (CDI) diagnosis, treating patients and dealing with recurrences is not straightforward, but a comprehensive and well-rounded understanding of what is needed to improve patient care is lacking. This manuscript addresses the paucity of multidisciplinary perspectives that consider clinical practice related and healthcare system-related challenges to optimizing care delivery.

Methods: We draw on narrative review, consultations with clinical experts and patient representatives, and a survey of 95 clinical and microbiology experts from the UK, France, Italy, Australia and Canada, adding novel multi-method evidence to the knowledge base.

Results and discussion: We examine the patient pathway and variations in clinical practice and identify, synthesize insights on and discuss associated challenges. Examples of key challenges include the need to conduct multiple tests for a conclusive diagnosis, treatment side-effects, the cost of some antibiotics and barriers to access of fecal microbiota transplantation, difficulties in distinguishing recurrence from new infection, workforce capacity constraints to effective monitoring of patients on treatment and of recurrence, and ascertaining whether a patient has been cured. We also identify key opportunities and priorities for improving patient care that target both clinical practice and the wider healthcare system. While there is some variety across surveyed countries' healthcare systems, there is also strong agreement on some priorities. Key improvement actions seen as priorities by at least half of survey respondents in at least three of the five surveyed countries include: developing innovative products for both preventing (Canada, Australia, UK, Italy, and France) and treating (Canada, Australia, and Italy) recurrences; facilitating more multidisciplinary patient care (UK, Australia, and France); updating diagnosis and treatment guidelines (Australia, Canada, and UK); and educating and supporting professionals in primary care (Italy, UK, Canada, and Australia) and those in secondary care who are not CDI experts (Italy, Australia, and France) on identifying

symptoms and managing patients. Finally, we discuss key evidence gaps for a future research agenda.

KEYWORDS

***Clostridium difficile*, *Clostridioides difficile*, *C. difficile* infection, healthcare improvement, healthcare systems, patient pathway**

1. Introduction

Clostridioides difficile (*C. difficile*), is a bacterium that commonly colonizes the human large intestine (1, 2). *C. difficile* colonization is not typically harmful, as other bacteria in the digestive system suppress its growth. However, under certain conditions, such as with the use of antibiotics or following gastrointestinal surgery (1, 2), *C. difficile* can grow in its vegetative state, producing toxins that damage the intestinal epithelium. Toxigenic *C. difficile* infection (CDI) can cause a range of bowel problems such as diarrhea, nausea and abdominal pain, and other symptoms like fever and loss of appetite (2). More severe CDI can cause complications such as pseudomembranous colitis, septic shock and death (1, 3, 4). The European Centre for Disease Prevention and Control estimates that CDI has a 4% mortality rate (5), which is higher in those who are frail, hospitalized (including in intensive care units) and/or elderly (1, 6, 7). However, the 4% mortality rate may be an underestimation given the challenges in attributing death directly to CDI. For example, 2020/21 data from England suggests that the 30-day all-cause fatality rate of CDI is closer to 13% (8).

Some aspects of the burden of CDI are relatively well understood, such as healthcare costs and mortality rates (9–12), but further research and validation is needed on the challenges faced by clinicians and patients in arriving at a CDI diagnosis, accessing treatment options and managing infections, including dealing with recurrences.

In this paper, we identify and reflect on the diverse requirements for effective clinical care for patients with CDI. As a foundation, we provide a brief overview the patient care pathway and explore variations in practice. We discuss challenges and key improvement needs as they relate to the care pathway as well as the wider healthcare system which frames clinical care. In doing so, we address the lack of multidisciplinary research that considers both clinical practice related requirements associated with diagnosis, treatment, ongoing patient monitoring, management of CDI recurrence and healthcare system influences on patient care, for example those related to access and organization of services, guidelines and regulation, and education and awareness raising (for patients and clinicians).

We focus specifically on patient care (i.e., diagnosis, treatment of initial CDI, patient monitoring and dealing with recurrence) and offer multidisciplinary and comprehensive insights drawn from a multi-method approach that tackles the often piecemeal nature in which challenges to patient care are at times researched. We consider the whole care pathway and the healthcare system that frame it. We recognize that infection prevention and control in hospital and community environments is also an important aspect of CDI management given that CDI is a frequent cause of healthcare-acquired infection (13), but this is discussed in numerous other literature and not covered in the scope of this study.

2. Materials and methods

This study involved conducting a narrative literature review, consultations with clinical experts and patient representatives, and a survey of clinical experts that sought to inform priorities for improvement in practice and key evidence gaps in need of further research.

The study focused on reviewing evidence from high-income countries (HICs), with a particular emphasis on the United Kingdom (UK), Italy, France, Canada, and Australia. These countries were selected given their geographical variety and all having a public healthcare system free at the point of service.

2.1. Narrative review

We conducted a narrative review following principles of rapid evidence assessment (REA) methodology (14). This includes: (1) development of a systematic search strategy, inclusion and exclusion criteria, and running the literature search; (2) screening the titles and abstracts of articles against the criteria; (3) prioritizing articles for inclusion in consideration of topic coverage, comprehensiveness, geographical focus and publication year; and (4) a full-text review and analysis of prioritized articles.

Two searches in PubMed were conducted in May 2021. The first focused on identifying relevant literature from the five case example countries (Australia, Canada, France, Italy, and the UK) and covered a 10-year timeframe (2011–2021). We also conducted a supplementary second search to identify additional literature from HICs more widely and focused on most recent review articles from the past 5 years (2016–2021), to fill gaps in literature on case example countries. A web-based gray literature search (June 2021) complemented the academic literature search and helped identify regulations and guidelines on CDI patient care in the case example countries. Twenty-nine papers were included (see **Supplementary material** for PRISMA flow diagram). We also considered some additional publications on specific points of detail raised in the literature that is included in the narrative review, where this was merited to provide further clarity on specific issues related to context or updates in regulation for example.

2.2. Consultations with clinical experts and patient representatives

We conducted consultations with leading CDI clinical experts and some patient representatives from the case example countries to refine, nuance and enrich insights from the literature and address

gaps in the existing evidence base. This allowed us to gain valuable experiential knowledge of diagnosing, treating and managing CDI and associated challenges. Consultations took the form of in-depth, semi-structured interviews and structured workshops (August – December 2021). With informed consent, eight one-to-one semi-structured interviews were carried out by the research team (LH and SS) with three experts from Canada, and one from Australia, the UK, France, Italy and a representative of a global foundation. Interviews followed established qualitative research methods used in health services research (15). Interview evidence is referenced with Int X, with X being the code number for an individual interviewee. To preserve anonymity in some instances where there is a direct risk of identity disclosure, and in line with informed consent, we withhold an interview reference number.

In addition, over the course of three online workshops (September 2021) health services research experts from RAND Europe (SM, LH, RR, and SS) and clinical and patient representative co-authors (MW, NP, TS, PG, GA, and JD) met in small groups/individually with the research team to enable knowledge-exchange and reflection on learning from the narrative review and interviews.

2.3. Survey of clinical and other experts on CDI

An online survey engaged experts from the five case example countries to explore views on needed priority actions for improving the care pathway for patients with CDI. The survey was designed based on findings from the earlier narrative review and consultations, with thematic analysis informing its structure and organization. The survey had subsections on different overarching areas of improvement (diagnosis and treatment; access and organization of service delivery and quality of care; guidelines and regulations; education and awareness raising for patients; and education and awareness raising for clinicians). These themes were developed based on the narrative review and consultation data, and in discussion with clinical expert and patient representative co-authors.

Within each thematic area, as part of the survey, respondents were asked to select improvement actions which they considered most important. The number of improvement actions selected as most important was dependent on the number of actions available to select from – respondents were asked to select actions within a top third threshold. For example, if six improvement actions were available, respondents were asked to select up to two they thought were most important. Respondents were also asked to share views on the most important gaps in evidence that need to be filled to inform future research and improvement. They were also asked to provide information on the nature of the CDI patient care pathway in terms of a patient's first point of contact with the healthcare system and in terms of referral practices (see the **Supplementary material** for the survey tool). The survey was disseminated via national and international networks and professional societies. It was open from January to May 2022 to allow sufficient time for respondents, many of whom were also involved in efforts to respond to the COVID-19 pandemic. All survey respondents participated with informed consent.

2.4. Analysis and synthesis

The findings across the narrative review and expert consultations were analyzed thematically, triangulated and synthesized by the research team to develop a multifaceted understanding of CDI diagnosis, treatment and management pathways across the countries of interest and associated challenges in patient care. This enabled us to develop the survey questions focusing on exploring priorities in terms of key areas in need of improvement in patient care. The survey was analyzed using the SmartSurvey analysis export tool and Excel, using a thematic approach. Survey analysis considers both similarities and differences in findings across the participating countries.

2.5. Ethics

This study involved a literature review, interviews with clinical experts and patient representatives, and a survey of clinical experts. The research was conducted in accordance with the Declaration of Helsinki. It was judged to pose minimal risks to participants and not to require ethical approval. It was reviewed retrospectively by the RAND Human Subjects Protection Committee and determined to be exempt under 45 CFR 46.104(d)(2)(ii), and, although exempt, the study's procedures and materials were found by the committee to be consistent with all rules laid out under 45 CFR 46 for the conduct of non-exempt human subjects' research. All participants gave informed consent and were provided with participant information sheets as part of this process.

3. Results

3.1. Survey respondents

We received 95 eligible responses to the survey. This includes 38 responses from Italy, 25 from UK, 16 from Australia, 12 from Canada, and 4 from France. While efforts were made to share the survey with relevant associations in France, some declined to engage due to CDI not being their core current focus. Given the low number of responses from France in particular, care should be taken when interpreting the survey findings presented at country level.

The majority of respondents stated infectious diseases as their primary area of work (61%), but there was also input into the survey from other clinical areas (e.g., gastroenterology and primary care) and from microbiologists. Most respondents identified as physicians/medical doctors (82%), but a variety of views were gathered, including for example from nurses (10%). See **Supplementary material** for further information on the demographics of respondents.

3.2. The clinical care pathway and associated challenges

The patient pathway for CDI involves key stages spanning diagnosis, treatment of initial CDI, patient monitoring and follow-up, and management of recurrence. Many aspects of care are similar across the case example countries, but there are also some important

differences (see Tables 1–3). In this section, we discuss the main aspects of the care pathway and associated challenges to optimizing patient care, drawing on evidence from the review of the literature and consultation with experts.

3.2.1. The diagnosis pathway

The diagnosis pathway for the example countries is outlined in Table 1. Diagnosis involves deciding if a test for CDI is required based on clinical signs such as diarrhea, abdominal pain or distension, ileus, and toxic megacolon [(2, 16, 17) Int1,7]; deciding which test to use; performing the test and interpreting results. Testing is only recommended on symptomatic patients as *C. difficile* can be present in the digestive systems of healthy people (2, 4).

Where diagnosis takes places varies, in part depending on whether the patient presents to primary care physicians in the community or in hospital (including emergency department), which in turn can depend on how unwell a patient is, with more severe cases more likely to be identified in hospital (Int2, 4–7).

In most case example countries, survey data suggests that the first point of contact with the healthcare system for the majority of patients with *community acquired* CDI is a primary care professional (92% of survey respondents in the UK conveyed this to be the case, 88% in Australia, 67% in Canada and 61% in Italy). However, this was not the case for France, where 25% of respondents reported that gastroenterologist experts in an outpatient hospital setting were the primary point of contact (although only four respondents were from France). Other primary points of contact identified by survey respondents ranged from emergency care settings to community-based infection prevention and control teams. However, some survey respondents felt that there was not one predominant point of contact, and this is likely to reflect diverse practices regionally, diversity between healthcare systems and differences related to variety in patient symptoms.

For patients with *hospital acquired* CDI in Australia, Canada, the UK, and France, the first point of care for patients tends to be the person under whose care they are more generally (81, 75, 80, and 50% of survey respondents, respectively). However, in Italy, this was only seen as the most common route by 40% of survey respondents. More common in Italy was referral to an infectious disease expert (47% of survey respondents), while this option was rarer in France, Australia, the UK, and Canada (25, 19, 16, and 8%, respectively). See Supplementary material for further information.

Who the patient will be referred to from the first point of contact in a community settings seems to vary both within and between countries, with patients being referred to either gastroenterologists, infectious disease experts and more rarely emergency care settings (see Supplementary material). Onward referral will depend on factors such as the severity of patient symptoms, parts of the country and preferences and personal experiences of the referring healthcare professional.

In terms of onward referral from inpatient/hospital admission settings, in Australia, Italy, and Canada this is most often to an infectious disease specialist in the inpatient setting (69, 74, and 58%, respectively). Less common is referral to other experts such as gastroenterologist, patients receiving referrals to multiple healthcare professionals at the same or to infection prevention and control nurses/teams (see Supplementary material for further detail).

Diagnostic testing can be done in public sector facilities or by private laboratories and this can vary both within and between countries, dependent on health system service organization and capacity (Table 1). For example, in Canada and Australia, most outpatient testing is conducted by private labs (interviewee reference numbers withheld to preserve anonymity) and while most hospitals have outpatient labs many patients live closer to private labs than hospital-based ones. In Canada, CDI testing does not require out of pocket payment by patients, including to private labs, with payment covered by central government funding (interviewee reference number withheld to preserve anonymity). Across the countries considered in this research, for patients who first present with symptoms in primary care, diagnostic testing is generally ordered by primary care providers (Int4–7). For patients who present with symptoms in hospital, diagnosis is generally overseen by hospital staff and specialists, such as infectious disease specialists and/or gastroenterologists (Int5–6).

The main diagnostic methods for CDI testing in patients of all ages are enzyme immunoassays [EIAs, to detect A/B toxin or the glutamate dehydrogenase (GDH) enzyme produced by *C. difficile*] and nucleic acid amplification tests (NAATs), with toxigenic culture and cell cytotoxicity assays (CCNA) also available (Table 1). Most diagnosis guidelines, including those for Europe, recommend a multiple step approach (Int1–3, 5, 7), combining EIA, NAAT, and toxigenic culture (e.g., to validate new tests) to improve diagnostic accuracy (2, 4, 16, 18–20). However, the specific combination recommended in the guidelines varies across countries and there is no clear diagnostic algorithm that applies universally (Int1–3, 5, 7). Table 1 provides additional detail based on expert consultation on which tests are conducted in case example countries, who conducts them, who pays for them and time to diagnosis.

3.2.2. Challenges related to diagnosis

Diagnosing patients with CDI is challenging. There is no single test that is recommended for use alone, and the frequent use of multiple tests to arrive at a diagnosis has both time and cost implications [(2, 4, 20, 21), Int2–3, expert workshops]. In addition, laboratories within and across countries can apply diverse testing strategies due to different guidelines [(20, 22) Int2–3, 5, 7] and so there is a lack of standardized practice. There are also both advantages and disadvantages to individual diagnostic tools, related to accuracy, turnaround time and distinguishing colonization from toxigenic infection [(2, 4, 16, 20, 21, 23, 24), Int2–3].

The CDI can be underdiagnosed, overdiagnosed or misdiagnosed. Underdiagnosis can occur due to a lack of clinical suspicion, for example in younger patients or when stool does not indicate CDI, or due to diagnostic methods that are not optimal (20, 22, 25). Decisions to order CDI diagnostic tests are often influenced by patient-profile related factors (rather than symptoms alone) and the type of setting a clinician is based in (22). Clinicians in hospitals with infectious disease specialists are more likely to conduct testing for CDI than those in general hospitals, due to differences in skills and training. This can contribute to underdiagnosis (22). On the other hand, for some diagnostic tests, positive results do not always directly correlate with clinical presentation and can lead to overdiagnosis (20, 26, 27). False positive rates can also contribute to overdiagnosis (28). Performance management incentives can also have unintended consequences for overdiagnosis in light of healthcare professionals in some countries requiring permission to send samples for *C. difficile* testing (expert workshops). Some hospitals have a requirement to test for CDI in all inpatient diarrhea cases which can lead to overdiagnosis, particularly if EIA's are used for diagnosis, due to their higher positive predictive value (Int2–3, 5).

TABLE 1 Symptom presentation and diagnosis.

Country	Which tests are conducted, and in what order?	Who conducts tests?	Who pays for diagnostic tests?	How long does it take to receive a diagnosis?
UK	Glutamate dehydrogenase (GDH) [or polymerase chain reaction (PCR) test for toxin gene] screening test plus toxin test. If GDH or PCR is negative, no toxin test is needed. If GDH or PCR is positive and toxin test is positive, this indicates CDI. Some labs may add a PCR test if GDH positive, but toxin test negative to provide infection control information.	Hospital lab.	Centrally funded.	Outpatient: 1–2 days, depending on sample transport time. Inpatient: 4–12 h, depending on lab processing.
France	Culturing to confirm if *C. difficile* is present. If this is positive GDH and EIA for toxin. A positive diagnosis can be confirmed if GDH and toxin tests are positive. If results are ambiguous, direct toxin by PCR can be conducted.	Outpatients: private labs. Inpatients: hospital or private labs.	65% of cost is centrally funded; rest to be paid by patient depends on their situation (long disease/other chronic conditions can be 100% reimbursed).	Outpatient: around 5 days. Inpatient: 2 days.
Italy	In many laboratories the confirmatory test is EIA. Some labs use NAAT for molecular detection of *C. difficile* toxin genes.	Hospital and private labs (no difference between outpatient and inpatient).	Inpatients: centrally funded. Outpatients: some out of pocket expenses for patient with the remainder covered by the national health service. Private tests paid for by patients.	Outpatient: up to 5 days (usually 2 days). Inpatient: 5 days (usually 2 days).
Canada	PCR alone or PCR and toxin testing (in any order).	Usually private labs, but can be hospital labs.	Centrally funded.	Outpatient: 1–2 days (depending on how quickly patient seeks care. The more severe cases presenting to emergency departments get diagnosed immediately). Inpatient: within 1 day.
Australia	Dependent on lab – some use PCR, followed by toxin tests if required, others still use antigen testing alone and others PCR alone.	Dependent on where patient presents - public hospital system has public lab, private hospitals use a range of private labs.	Usually funded publicly or paid for by health insurers.	1–2 days for both inpatients and outpatients.

Misdiagnosis may occur when testing is performed after treatment, as *C. difficile* genetic material remain in stool weeks after infection resolves (4, 29). Complex patients, such as younger or older aged or those with co-morbidities, can also create challenges in reaching a CDI diagnosis due to difficulties in distinguishing *C. difficile* colonization from a toxigenic infection and when patients display unusual symptoms (Int2, 4, 7).

Patients can also face long waiting times for diagnosis, particularly if they present in the community, due to lack of availability of primary care physicians, physical distance from a lab, need to implement infection control measures in hospital, diarrhea being a non-specific symptom and multiple testing requirements [(2, 20, 29, 30); Int2, 4–8, expert workshops]. This can have implications for health outcomes (2, 20, 29, 30).

There are also challenges in classifying the severity of CDI, in part related to a lack of consensus on clinical markers for severity, and a reliance on clinical judgment (4, 29, 31, 32).

3.2.3. Treatment of first episode CDI

Antibiotics are the main treatment used for CDI. The antibiotics used are primarily oral vancomycin, fidaxomicin and metronidazole. Vancomycin and fidaxomicin have similar efficacy (2, 4, 16, 18, 31, 33–38) and are recommended in The European Society of Clinical Microbiology and Infectious Diseases guidelines [(2, 4, 18, 33, 34, 38); Int3]. While metronidazole has traditionally been the first line treatment in the past, most countries appear to be replacing this with vancomycin and/or fidaxomicin as these have demonstrated higher efficacies for CDI. However, it is still used in some situations (2, 4, 16, 18, 19, 31–33, 35, 36, 38–42). While the choice and combination of antibiotic options vary according to national guidelines, treatment options can also vary within countries. For example, each province in Canada has its own treatment guidelines (expert workshops). The choice can also be influenced by cost considerations, e.g., fidaxomicin may not be offered as a first option in some contexts as it is more expensive (Int2–3, expert workshops).

Non-antibiotic-based treatments for initial CDI are also available for use as add-on treatments to an antibiotic regime. Surgery can be used to treat severe or fulminant CDI (2, 18, 33, 35, 40, 43). Monoclonal antibody therapy, such as bezlotoxumab, is emerging as a potential treatment that may be effective at preventing recurrences of CDI (2, 4, 18, 33, 36). Probiotics are rarely used as part of the process of treating CDI and are not recommended in guidelines given the evidence on efficacy is limited (2, 16, 29, 35, 36).

After the diagnosis of CDI, it is important for patients that any non-CDI focused antibiotic therapy or proton pump inhibitors are stopped, if possible, to prevent worsening of the infection (2, 16, 17, 31, 34, 35, 43).

Treatment decisions can be made by diverse healthcare professionals. In some countries, this is often by primary care physicians who can be the first point of contact for the patient,

TABLE 2 Treatment of initial infection, and patient monitoring and follow-up.

Country	How quickly does treatment commence?	How is treatment initiated and by whom?	Patient involvement in treatment decision-making	Treatment course for initial, mild infection	Treatment course for initial, severe infection	How long does treatment typically last?	Who pays for treatment?	How are patients monitored?	How are patients assessed for cure (if at all)?
UK	Same day as diagnosis.	Larger hospitals: specialists determine treatment regime (infectious disease specialists, gastroenterologists). Smaller hospitals: primary physician. Outpatient: primary care (possibly with advice from microbiologists based on test results).	Little involvement in initial infection. Greater involvement for recurrences (particularly FMT).	Metronidazole (or vancomycin).	Vancomycin (or fidaxomicin for high recurrence risks). Alternatives are: high dose vancomycin (with/without IV metronidazole), rifampicin or IV immunoglobulin. Life threatening infection treated with nasogastric/rectal vancomycin, with/without IV metronidazole.	10 days.	Centrally funded.	Outpatient: little-no monitoring. Inpatient: regular monitoring of stool, bowel movements and lab tests. Involves microbiologists, infectious disease specialists, infection prevention/control staff and the primary physician.	Symptom resolution.
France	Same day as diagnosis (sometimes before confirmation of diagnosis).	Generalist or practitioner.	Yes, always.	Fidaxomicin.	Vancomycin or fidaxomicin.	10 days.	65% covered for all patients; rest to be paid according to patient situation.	Mainly resolution of diarrhea.	Symptom resolution.
Italy	Same day as diagnosis	Primary physician (with GI or infectious disease specialist support if non-specialists) for in and outpatients.	Little involvement in both initial and recurrent infection.	Vancomycin or, less commonly, fidaxomicin.	Vancomycin (increasing use of fidaxomicin following updated ESCMID guidelines).	10 days.	Centrally funded.	Outpatient: little-no monitoring. Inpatient: monitored by ID/gastroenterologists (e.g., bowel movements, blood tests).	Symptom resolution.
Canada	Same day as diagnosis (for inpatient and outpatients).	Outpatient: primary care. Inpatient: primary physician (with input from pharmacist or, in fulminant/failure to respond cases, specialists).	Little involvement in initial infection. Greater involvement for recurrences (particularly FMT).	Vancomycin (can be metronidazole or fidaxomicin if available).	Vancomycin (or fidaxomicin if available). Severe and complicated infection primarily treated with vancomycin with IV metronidazole.	10–14 days (typically 10 days for the first episode).	Outpatient: out-of-pocket payment for vancomycin and fidaxomicin, but not metronidazole (although this varies by province). Inpatient: centrally funded.	Outpatient: little-no monitoring. Inpatient: regular monitoring of stool, bowel movements and lab tests. Fulminant cases involve infectious disease specialists, gastroenterologists and/or surgical staff.	Symptom resolution.
Australia	Inpatient: same day as diagnosis. Outpatient: 3–5 days.	Primary physician (with GI input in severe cases) for both in and outpatient.	Little involvement in initial infection. Greater involvement for recurrences (particularly FMT).	Metronidazole (or vancomycin, fidaxomicin).	Vancomycin with IV metronidazole is first line, second line is nasogastric vancomycin and IV metronidazole, with/without rectal vancomycin. FMT in refractory infection.	10–14 days	Subsidized by Medicare.	Outpatient: little-no monitoring. Inpatient: regular monitoring of bowel movements and lab tests. Ideally involved infectious disease specialists.	Symptom resolution.

10.3389/fmed.2022.1033417

TABLE 3 Preventing and managing recurrences.

Country	What are the approaches for preventing recurrences?	How are recurrences diagnosed?	What is the treatment course for initial recurrence?	What is the treatment course for 2+ recurrences?	Who pays for treatment for recurrences?
UK	Fidaxomicin or monoclonal antibody therapy (although with limited use due to cost).	Same as initial infection.	Fidaxomicin (or vancomycin if cost is issue).	Fidaxomicin (if not used for initial infection); tapered/pulsed vancomycin; IV immunoglobulin; or FMT.	Centrally funded.
France	Antibiotic stewardship and specific selection of antibiotics that will minimally alter the normal anaerobic microbiota.	Same as initial infection, but faster; often with primary care physician (for outpatients).	Fidaxomicin.	Vancomycin with tapering of doses or FMT.	Antibiotics: 65%+ of fidaxomicin is centrally funded depending on coverage. For FMT, there is a different legal framework; payment is taken care of but modalities differ center by center; the assessment cost is paid for according to the centers. Depending on the commission of the establishment/hospital.
Italy	Fidaxomicin, taper/pulse regime of antibiotics, monoclonal antibody therapy.	Same as initial infection.	Generally vancomycin (increased trend of fidaxomicin if the initial treatment was done with vancomycin). If the initial treatment was done with fidaxomicin, generally bezlotoxumab is used for preventing further recurrences.	Tapered vancomycin, fidaxomicin, monoclonal antibodies, and FMT if available.	Centrally funded.
Canada	Tapered vancomycin can be used in high recurrence risks (although rarely for first episodes).	Same as initial infection or reliance on clinical presentation.	Vancomycin (very high risk patients may have tapered dose of vancomycin).	Tapered dose of vancomycin, or FMT.	Inpatient: hospital funding structure. Outpatient: the patient.
Australia	Ceasing use of contributing antimicrobials, taper regime of vancomycin, FMT.	Same as initial infection.	Vancomycin.	Vancomycin with/without taper, fidaxomicin, rifaximin chaser or FMT.	Medications are funded through the pharmaceutical benefits scheme. For inpatients, medication would be centrally funded. FMT conducted in public hospital will be covered by public hospital funding structure.

but for patients presenting with symptoms in hospital settings infectious disease specialists or gastroenterologists are often involved in deciding on the treatment approach (Int2, 5–7). In some countries, pharmacy staff can also be involved (Int2–3). According to interviewees across countries, patients generally have little involvement in deciding what treatment they will receive (Int3, 5–7). However, patients may have more involvement in decision-making for recurrent infections, particularly in the use of FMT (Int4–5, 7). According to one expert, the extent of patient involvement in treatment decision-making is also dependent on how receptive the clinician is to this, and how unwell the patient is (with sicker patients potentially being less involved in decision-making) (Int4).

Table 2 provides additional detail on treatment pathways, based on expert consultation, elaborating on how quickly post-diagnosis treatment commences, how treatment is initiated and by whom, whether patients are involved in treatment decision-making, the treatment course and duration and who pays for treatment in case example countries.

3.2.4. Treatment challenges

Ensuring appropriate and effective treatment that is optimal for an individual patient comes with a set of challenges. For example, anti-CDI antibiotics are the first-line treatment for CDI, but can have side-effects such as a further imbalance of the gut microbiome [(2, 7, 16, 23, 33, 36, 44, 45); Int1, 3, 5–6]. Although the evidence base is inconclusive, there is also some concern about risks of resistance to mainstream therapies (18, 33, 36).

Timely treatment matters for successful outcomes, but there can be challenges to ensuring timely treatment as well. Although these appear rare (29) they are a risk, especially if diagnosis is not timely. Patients with additional complexities, such as the elderly and patients with co-morbidities, may face difficulties in treating their CDI due to frailty, multiple health issues that need addressing or a lack of response to treatment (Int2, 5).

There is a lack of evidence on the optimal treatment regime for CDI (7, 16, 33), especially for severe infections (7) and cost considerations may also play a role in what is used (as we expand on in section "3.3.3 Economic considerations").

3.2.5. Patient monitoring and follow-up

If a patient is diagnosed in the community, there is generally little follow-up across case example countries, and patients are told to return to their GP if their symptoms do not resolve (Int2, 5–7), given that in most cases infection may be mild.

Patients in hospital (either with initial or recurrent infection) are subject to closer monitoring, which primarily involves referencing stool charts, recording bowel movements, testing for white blood cell counts, assessing inflammatory marker, and, in more complex

cases, CT imaging (Int2, 5, 7). This is to check for compilations such as severe dehydration, acute kidney injury, fever, ileus, and toxic megacolon (23) and side effects of medication.

Monitoring of inpatients can involve a diverse range of healthcare professionals and varies across countries. For example, in England, guidance states that effective patient care should involve weekly monitoring by a multidisciplinary team of healthcare staff, including microbiologists, infectious disease or infection prevention and control clinicians, nurses, a GI or surgeon, a pharmacist, and a dietician (31). According to interview evidence, these teams may be more frequently in place for more complex cases, such as older patients or those with underlying conditions. In Australia, ideally infectious disease specialists are primarily involved monitoring diagnosed inpatients and in Canada, data from interviews suggests specialists would not be consulted for the first CDI episode, unless it was a fulminant case which would involve gastroenterologists, infectious disease specialists and/or surgery teams (interviewee reference number withheld to preserve anonymity).

Table 2 provides additional detail on how patients are monitored and how they are assessed for cure in case example countries, based on expert consultation.

3.2.6. Monitoring related challenges

Monitoring patients with CDI in hospital can be difficult as bowel movements are not always easy to record due to lack of available staff or due to a threshold of 3+ loose bowel movements over 24 h for a patient to be tested for CDI (Int5). Staff capacity constraints are the key challenge.

It can be difficult to ascertain whether a patient has been cured and whether an episode has been resolved. Some literature suggests that an initial CDI episode can be considered as 'cured' if symptoms resolve after 30–90 days (18), but there is a lack of consensus on this matter and toxins and genetic material from *C. difficile* can remain in the stool for several weeks after the infection is treated (16) (expert workshops).

3.2.7. Managing CDI recurrence

A review by Khanna (23) states that CDI recurrence occurs in an estimated 20–30% of cases after the first CDI episodes, increasing to approximately 60% of cases after three or more episodes (23). Should CDI recurrence be suspected in a patient of any age, it is important to distinguish whether it is actual recurrence or if symptoms are due to something else, such as post-infection irritable bowel syndrome (IBS) (23). While distinguishing between recurrence and an entirely new CDI infection is also important as treatment regimes can vary, it can be difficult to achieve this in practice. Diagnosing recurrence generally involves first an assessment of symptoms and then diagnostic testing [(23, 34); Int1–3, 5, 7].

Treatment options for recurrent CDI are more diverse than for first episode infection and include therapies such as fecal microbiota transplantation (FMT), antibiotics different to those given in the initial infection such as vancomycin or fidaxomicin (if not used first time) stronger doses of antibiotics than those used for the initial episode and taper-pulse antibiotic regimes. Alternative antibiotic regimes, noted by multiple articles, are: (1) fidaxomicin; (2) taper–pulse vancomycin; (3) vancomycin or fidaxomicin followed by FMT; and (4) vancomycin followed by rifaximin (for multiple recurrences where alternatives have failed) [(2, 4, 16, 18, 29, 31, 33, 35–38, 40, 44); Int1–5, 7]. Rifaximin is recommended for patients who cannot undergo FMT (37). Metronidazole is not recommended for treatment of recurrent infections (18, 33, 35). Patients with risk factors, but FMT failure can undergo a course of antibiotics and FMT can be re-considered should recurrence occur (23). **Table 3** elaborates on approaches to preventing recurrence, the diagnosis of recurrence, the treatment course and who pays for treating recurrences in case example countries, drawing on expert consultation.

3.2.8. Challenges in managing recurrence

The CDI recurrences can be challenging to diagnose due to lack of monitoring for recurrence symptoms and difficulties in distinguishing recurrence from new infection [(2, 42, 45); Int2–3, 5, 7].

There are also challenges in both access to and efficacy of some treatments, for example FMT. FMT efficacy for treating recurrent CDI can be influenced by factors such as having an underlying condition [such as IBS or Irritable Bowel Disease (IBD)], the use of systemic antibiotics after FMT and being hospitalized (18, 23, 44). While FMT is generally considered safe, there are some risks of adverse events (such as abdominal discomfort, nausea, vomiting, transient diarrhea, and aspiration), infection transmission and post-infection IBS. There are also some concerns about the lack of research into long-term safety [(2, 16, 18, 23, 33, 36, 44); Int5]. Workforce capacity, facilities and resource challenges can also have an impact on access to FMT (Int2, 6).

3.3. Influences on patient care related to the wider healthcare system and associated challenges

Diverse features of the wider healthcare system, related to (i) access and organization of service delivery and quality of care; (ii) guidelines and regulation; (iii) economic considerations; (iv) education and awareness raising of healthcare professionals; (v) education and awareness raising for patients, and (v) COVID-19 pandemic related factors influence the care of patients with CDI. **Supplementary Table 2** summarizes the key challenges applying to the case-example country contexts.

3.3.1. Access and organization of service delivery and quality of care

The organization of healthcare services for patients with CDI, such as links between primary and secondary care, the set-up of outpatient care and availability of specialist CDI clinics, can influence the type of care CDI patients receive (Int3–5). The degree of multidisciplinary work may also vary, which may influence the management of some patients with CDI (expert workshops).

Access to treatments such as FMT are also a complex challenge (as introduced earlier) (36, 37). Identifying, recruiting and retaining stool donors, challenges to staff capacity and delivery facilities, lack of standardization of screening of donors, costs of testing donors and the emergence of new pathogens that need to be tested for all present access challenges [(18, 23); INT2, 4–7]. Beyond access, the lack of standardization of FMT procedures and a need for further evidence on optimal stool preparation procedures and modes of FMT delivery (e.g., colonoscopy, enema, and capsules) can also represent barriers to optimal patient care and experiences (18, 23).

3.3.2. Guidelines and regulation

Guidelines for diagnosis, treatment and management of CDI can vary across countries. For example, some guidelines have different recommendations for who could be at higher risk for CDI and who should be tested for the infection, e.g., the recommended 2–3 step algorithm for testing for CDI can differ across country guidelines (43) (expert workshops).

There is also variation in guidelines on what to use as first-line treatments for CDI across countries (33), for example whether to use metronidazole. To illustrate, Canadian guidelines only recommend this in specific situations (e.g., for children, where vancomycin/fidaxomicin are not available or cannot be used) whereas Australian guidelines recommend metronidazole as a first line treatment in initial (mild) infections (31, 35, 40). Older guidelines are still more likely to recommend the use of metronidazole than more up to date ones, as well as to not include fidaxomicin as a key treatment option. However, the European Society of Clinical Microbiology and Infectious Diseases guidelines, updated in 2021, do now recommend the use of fidaxomicin as a first line treatment for initial CDI (38) but the extent to which this is reflected in individual in-country practices remains to be seen.

While guidelines on preventing and treating recurrences of CDI appear slightly more consistent across case example countries, there is still variation in the recommendations made, particularly for treating first recurrence (31, 35, 37, 40, 43). For example, 2018 Canadian and 2016 Australian guidelines recommend the use of vancomycin to treat first recurrences in adults, but 2021 English guidance recommends fidaxomicin (31, 35, 40). The Australian guidance also recommends the use of a rifaximin chaser after 2+ recurrences in adults, but this is not included in the reviewed English or Canadian guidelines (31, 35, 40). Differences in guidelines for managing recurrences may be related to factors such as new evidence emerging over time (which can be incorporated into newer guidance, but not always in a timely manner) or accumulation of evidence of one treatment being inferior (37), and wider availability and reimbursement contexts may also play a role.

While guidelines may be in place to support the treatment and management of patients with CDI in many countries, evidence suggests that these are not updated on a regular basis (Int3, 5, 7). This is a challenge to optimizing care quality. A European survey study published in 2018 noted that while national guidelines for managing patients with CDI were present in 14 (70%) of the included countries, 4 countries had not revised the guidelines within the last 5 years (34).

Guidelines are also often modified or applied inconsistently in clinical practice (7, 19, 30, 39). There may be good reasons for doing so, but this merits further research. For example, Turner et al. (30) note that there can be a risk of clinicians prescribing treatment for CDI based on a positive result from a single test, as opposed to the recommended 2–3-step algorithm of multiple tests (30). A lack of adherence to guidelines may also be in part affected by a lack of auditing practices on adherence (34) or due to a lack of a local policies and protocols on CDI treatment (39). Although meriting further research, not all clinicians necessarily read updated CDI guidelines in detail due to their length (expert workshops).

3.3.3. Economic considerations

Several financial resource related considerations can have an impact on the care of patients with CDI and give rise to challenges. The cost of some anti-CDI antibiotics may be difficult for healthcare systems to absorb (2, 33) and this may also be a challenge in relation to emerging treatments, e.g., monoclonal antibody therap[illegible] (workshops). Fidaxomicin is more costly (in terms of acquisition) compared to metronidazole and vancomycin, which may influence its availability in some settings (34, 36, 40, 45), despite some emerging evidence suggesting that fidaxomicin is more cost-effective than other antibiotics for both initial and recurrent CDI in most situations due to the reduced risk of recurrence, despite higher upfront cost (46).

While data on the cost of recurrence is more limited compared to initial infection, evidence from two articles suggests that recurrent CDI costs more to treat than initial infection, likely due to higher severity and longer lengths of hospital stay (46, 47). For example, a 2018 study using data from 45 patients from the UK indicated that length of stay for patients with recurrent infection was 33 days, significantly longer than the 17 days for those with initial infections (47). Intensive care unit stays were also found to be longer for patients with recurrent infections compared to initial infections in this study (2.5 vs. 0.7 days, respectively) (47). Treatment, pathology tests, sterile services, linen, medical pay and overheads have also been found to cost more in recurrent CDI compared to initial infection (47). FMT is generally considered to be cost-effective for treating recurrent CDI (46, 48), but there is a need for further research on how wider healthcare systems factors such as setting up and maintaining stool banks may impact on cost effectiveness.

Cost can be a barrier not only to optimizing treatment, but also to optimal diagnostic test use and may contributes to some of the variation seen in CDI guidelines across countries (Int2–3, 5–6, expert workshops).

In some countries (e.g., Canada), reimbursement for treatment varies across provinces which impacts on efforts to standardize practices at a national level and results in subsequent variation in treatment regimens (expert workshops).

Litigation costs and hospitalization costs can also present financial challenges (expert workshops) (49).

3.3.4. Education and awareness raising for patients and clinicians

Patient-related issues such as stigma, disgust and embarrassment or low awareness and understanding of CDI symptoms can be a barrier to timely diagnosis. This can lead to patients delaying seeking help from a healthcare professional or not providing all the information about their symptoms (e.g., appearance of bowel movements) (Int2, 4, 6, 8, expert workshops). A scarcity of public health campaigns (national and regional) about CDI symptoms and the importance of seeking care can also impact on access to the right care at the right time and place (expert workshops). It can also impact on resorting to treatments for which sufficient evidence may be lacking, such as probiotics (expert workshops).

There is limited evidence on the impact of CDI from the patient or care-giver perspectives, and this is an area that requires further research. One Canadian study explored the impact on patient's quality of life (QoL) as a result of CDI by conducting a survey of 167 people with CDI and their carers (29). QoL was ranked from 1 (patient is unable to care for self and requires hospital care) to 6 (patient can undertake normal day-to-day activities without support). The results indicate that those patients who report a lower QoL before CDI experience a larger impact on their QoL when they have the infection. Moreover, carers reported that patients had lower QoL scores than the patients reported about themselves (median QoL of 3 compared to [illegible] respectively).

Clinician awareness and knowledge of *C. difficile* diagnostic, treatment and referral processes can also be relatively low, especially in primary care and amongst some specialist clinicians such as surgeons across the case example countries (expert workshops). This is partly due to CDI not having prominence in the medical curriculum (or having not been there in the past) and lack of awareness of guideline updates (Int3, 5–8, expert workshops). The extent to which healthcare providers discuss bowel movements with patients in a way conducive to identifying a potential case of CDI can also influence whether or not a patient is tested for CDI (Int4, 6, expert workshops). There can also be risks from clinicians not interpreting test results correctly and treating a patient in cases where *C. difficile* has been detected but is not toxigenic (expert workshops).

3.3.5. Impact of COVID-19 on the CDI care pathway

Unforeseen events such as the COVID-19 pandemic can have an impact on access to care and care quality. In some contexts (e.g., the UK), data suggests that cases of CDI (particularly hospital acquired infection) rose during the pandemic and the 30-day case fatality rate for CDI also increased, and this has been associated with COVID-19 (8, 50). Although evidence of the impact of COVID-19 on the care of patients with CDI is currently scarce and inconclusive, consultations with experts noted that in some case example countries, the prioritization of dealing with COVID-19 increased risks related to timely diagnosis and treatment of patients with CDI symptoms (expert workshops, Int4–6) and that patients may have avoided seeking healthcare due to fears of contracting COVID-19 (Int8). In the UK and France FMT services were stopped by regulatory agencies during the early stages of the pandemic.

3.4. Priorities for improving the care of patients with CDI

Based on insights into the CDI care pathway and associated challenges outlined previously and as informed by the narrative review and stakeholder consultations, a survey was developed to explore priority areas where taking action could help improve patient care. Respondents across case example countries were asked to select which improvement opportunities they thought were most important within the following categories derived through thematic analysis of literature, interview and workshop data: (1) diagnosis and treatment, (2) access and organization of service delivery and quality of care, (3) guidelines and regulations, (4) education and awareness raising for clinicians, (5) education and awareness raising for patients. (In addition to selecting the most important opportunities-i.e., top priorities, respondents also rated opportunities. To avoid repetitiveness and in light of consistent messaging, our analysis focuses on the selection of the most important opportunities rather than rating data). The survey also explored evidence gaps that need addressing. Respondents were asked to select the top third threshold in terms of importance, amongst a list of actions in each thematic area (so that in a list of ten items, for example, they were able to select up to three, if there was a list of six, they could select up to two). Throughout, where presenting the findings, we highlight the actions where 50% or more respondents reported it as a priority area for improvement (as a threshold for strong agreement), but we also reflect on the wider sentiment across survey respondents

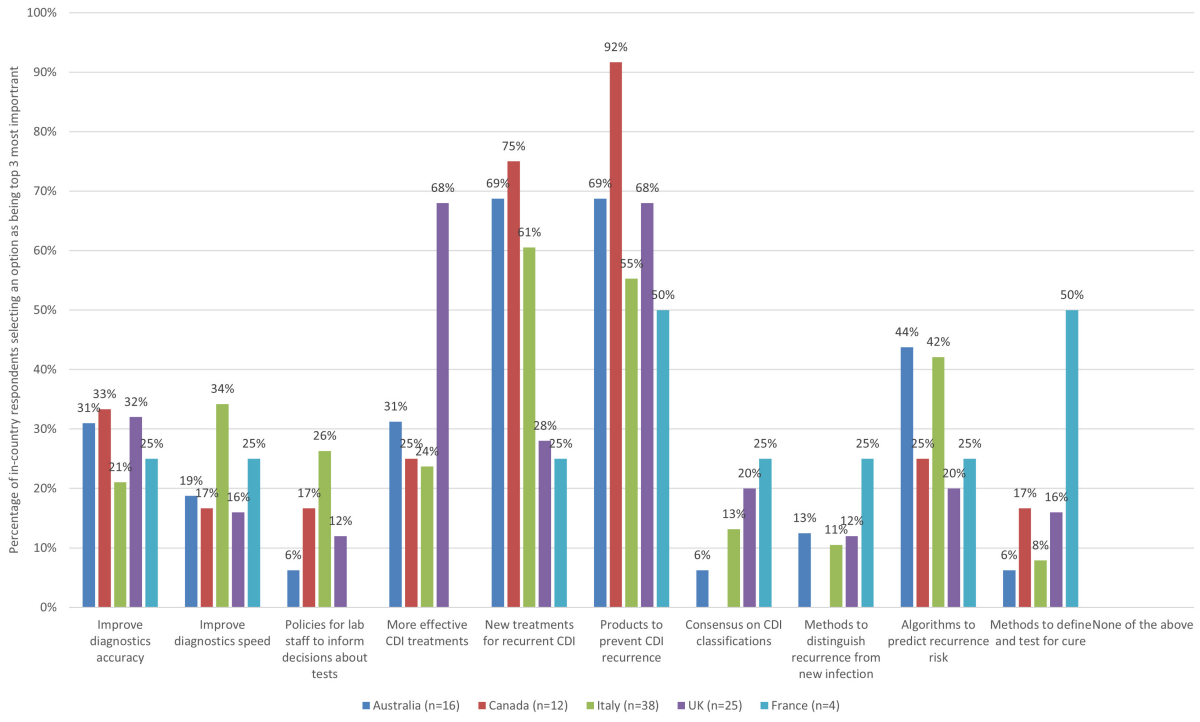

FIGURE 1
The improvement actions related to diagnosis and treatment of CDI that are the most important (bars represent the percentage of respondents that rated each improvement action as one of the most important by country).

(i.e., commenting on areas where 30% of more of survey respondents felt an action was a priority).

3.4.1. Diagnosis and treatment

Amongst a list of ten improvement actions related to diagnosis and treatment, there was strong agreement across all surveyed countries that innovative products for preventing recurrence of CDI was a key priority (see Figure 1). This option was selected by 92% of respondents from Canada, 69% from Australia, 68% from the UK, 55% from Italy, and 50% from France, although due to low response numbers, this equated only two respondents for France.

Developing innovative, more effective treatments for treating recurrent CDI was also seen as a key priority by respondents in three countries (75% Canada, 69% Australia, and 61% Italy). Although this option was seen as a top priority by some respondents from the UK and France as well (28% of UK and 25% of respondents from France), the relative strength of sentiment about its importance was lower across respondents in these countries.

When looking at country-level insights, some key findings are captured in Table 4.

3.4.2. Access and organization of service delivery and quality of care

As there were six improvement actions to select from in the theme of access and organization of service delivery and quality of care, respondents were asked to select up to two (top third threshold) improvement actions they thought were the most important, and thus represent priorities (Figure 2).

When looking across countries, there are both some similarities, but also notable differences and a variety of views on key areas in need of improvement as they relate to this theme. For example, addressing variation in access to FMT at local, regional and national levels was seen as a priority area for improvement by over half of all respondents from Canada and Australia (75 and 56%, respectively), but this was not the case for the UK (44%), Italy (32%), or France (0%). Half or more of respondents from Australia (63%) and France (50% – although this is only 2 respondents) saw improving timeliness of diagnosis as a top priority, and Italy was close (47%), but this was not the case for the UK (40%), or Canada (25%). Improving access to effective antibiotics for treating CDI was seen as a top priority for over half of respondents from Canada and France (58 and 50%, respectively), but this was not the case for Italy (37%), Australia (25%), or the UK (12%). Facilitating more multi-disciplinary care delivery in the management of patients with CDI was selected within the top improvement actions by half or more of respondents from the UK, Australia, and France (68, 50, and 50%), nearly half in Italy (47%), but much less in Canada (8%).

When looking at country level data, there are both similarities and differences in views about improvement priorities related to access and organization of services and quality of care. Although speculative and meriting further research, these may have to do with some differences in the way healthcare systems are organized in terms of provision of care to patients with CDI and unique challenges. See Table 5.

3.4.3. Guidelines and regulations

Amongst a list of nine improvement actions related to the theme of guidelines and regulation, respondents were then asked to select up to three (top third) they saw as most important (Figure 3).

When looking across the participating countries, it is notable that more than half of respondents from most surveyed countries saw updating diagnosis and treatment guidelines more regularly as a top

TABLE 4 Country-level insights relating to diagnosis and treatment priorities.

Country	Insights
Australia	• Strong agreement that developing more effective treatments for treating recurrent CDI and developing innovative products for preventing recurrent of CDI were top priorities (69% of respondents for both), mirroring findings from the survey overall. • Some other areas were also seen to be a priority, but by fewer than half of all respondents. For example, 44% of respondents from Australia saw developing algorithms to more accurately predict the risk of CDI recurrences as a top priority. Just under a third (31%) saw improving the accuracy of diagnostic methods and developing more effective treatments for recurrent CDI as top priorities. • Other options were selected more rarely (less than 30% of respondents).
Canada	• Two options stood out as key priorities (aligned with overall survey findings) with very strong agreement across survey respondents. Developing products to prevent recurrence was seen as a top priority by 92% of respondents. Three quarters (75%) saw developing new treatments for recurrent CDI as a key priority. • Improving diagnostic accuracy was seen as less of a priority, although it was still selected by one-third (33%) of respondents. • Other improvement areas were selected as priorities more rarely (less than 30% of respondents). • No respondents from Canada felt that there is a need for consensus related to classifications of CDI nor for improved methods to distinguish recurrence from new infection (unlike in other countries).
Italy	• Over half of respondents saw developing new treatments for recurrent CDI (61%) and products to prevent recurrence (55%) as key indicating strong agreement on these priorities (in agreement with views from Australia and Canada). • However, there was a variety of views, with all other improvement actions also seen as a top priority by at least some respondents and to varying degrees. For example, 42% saw developing algorithms to predict risk of recurrence as a top priority and 34% felt improving the speed of diagnosis is a key priority. • Other options were selected as priority more rarely (less than 30% of respondents).
UK	• There was strong agreement on the importance of two improvement areas (68% of UK respondents selected them as a top priority), these being developing more effective treatments for treating initial CDI and develop innovative products for preventing recurrence of CDI. • Interestingly, unlike in Australia, Canada and Italy, developing new treatments for recurrent CDI did not emerge as a top priority in the UK (selected by 28% of respondents). • Significantly fewer respondents saw other options as a top priority. While improving the accuracy of diagnostic methods was seen as a top priority by 32% of respondents, no other option was seen as a priority by 30% more of the UK respondents.
France	• There is no strong agreement on what the top improvement priorities within the diagnosis and treatment space are (this may partly relate to a low number of survey responses). • Six actions made it into the top third threshold in terms of priority actions. • Methods to define and test for cure was selected as a key priority by 50% (two out of the four) respondents from France.

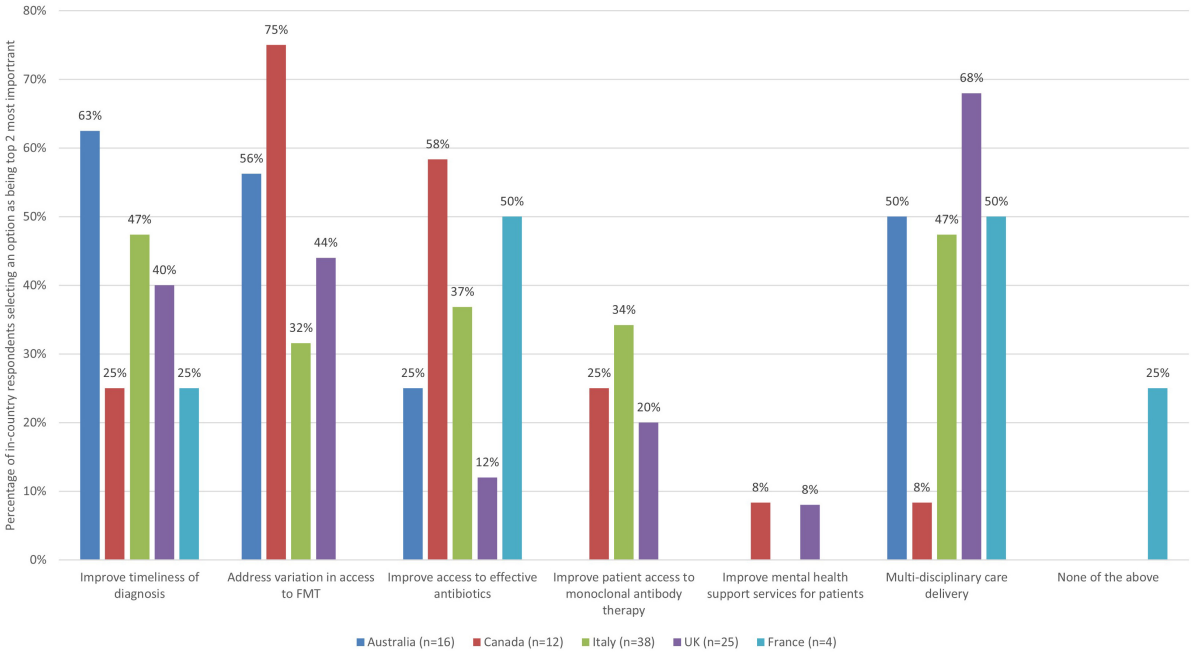

FIGURE 2
The improvement actions related to access and organization of service delivery and quality of care for CDI that are the most important (bars represent the percentage of respondents that rated each improvement action as one of the most important by country).

priority (69% for Australia, 67% for Canada, and 60% for the UK) and nearly half for Italy (47%), but this was not the case for France (25%, 1 respondent). More than half of respondents from Italy (63%) also felt that standardizing diagnosis guidelines within countries is of key importance, but this was not the case for other surveyed countries (44% Australia, 36% UK, 33% Canada, and 0% France). More than 50% of respondents from Australia (69%) felt that guidelines to help with standardization of FMT practice would be very important as well, but this was not the case for other countries (42% Canada, 40% UK, 25% France, and 5% Italy). Over half of respondents from France and the UK (75 and 56%) respectively felt that improving guideline clarity was a priority, but significantly fewer from Canada, Australia and Italy selected this as a top priority (33, 25, and 24%, respectively).

Considering country level data, some notable findings are presented in Table 6.

3.4.4. Education and awareness raising for patients

As there were seven improvement actions to select from in the theme of education and awareness raising for patients, respondents were asked to select up to two (top third) improvement actions they thought were the most important (Figure 4).

When looking at the responses across countries, a number of similarities emerge, but also some unique perspectives. For example, half or more respondents from all countries saw educating patients with CDI on the appropriate use of antibiotics as a top improvement action (68% Italy, 56% UK, and 50% for Australia, Canada, and France). Half of respondents from France and Canada, and nearly half of respondents from the UK (48%) and Italy (47%) also felt that educating patients with CDI about the management of the illness and the potential future impact on their lives was a priority area for improvement, but this was not the case for Australia (31%). Improving patient choice in relation to FMT was selected as a priority action by 50% of respondents from Australia, but by fewer than half of respondents from other countries (42% Canada, 40% UK, 11% Italy, and 0% France).

When zooming into country level data, some notable findings are presented in Table 7.

3.4.5. Education and awareness raising for clinicians

As there were seven improvement actions to select from in the theme of education and awareness raising of clinicians, respondents were asked to select up to two (top third threshold) improvement actions they thought were the most important (Figure 5).

There was clear agreement on the top two priority actions. In most countries, educating and supporting healthcare professionals in primary care was seen a top priority improvement action (68% of respondents from Italy, 68% from UK, 58% from Canada, and 50% from Australia), but this was not the case for France (25%, 1 respondent). Half or more of respondents from Italy (76%), Australia (69%) and France (50%, 2 respondents), and nearly half of respondents from the UK (48%) saw educating and supporting healthcare professionals in secondary care who are not experts regularly dealing with patients with CDI as a top priority, but this was not the case for Canada (25% selected as most important).

When zooming into country level data, some notable findings are indicating relatively strong alignment between views from different countries (Table 8).

3.4.6. Evidence gaps

Respondents were asked to select areas where there are particularly important gaps in evidence that need to be addressed to support evidence-based practice and high-quality care for CDI. As there were ten evidence gaps to select from, respondents were asked to select up to three (top third threshold)

TABLE 5 Country-level insights relating to access and organization of service delivery, and quality of care priorities.

Country	Insights
Australia	• Half or more of survey respondents saw improving timeliness of diagnosis (63%), addressing variation in access to FMT (56%) and improving multi-disciplinarity of care delivery (50%) as top priorities – signaling strong agreement on these issues. • No other option had more than 30% of survey respondents seeing it as a top priority. • No respondents from Australia saw improving mental health support for CDI patients as a priority.
Canada	• Addressing variation in access to FMT and improving access to effective antibiotics were seen as key proprieties (selected by 75 and 58% of respondents, respectively). • No other option received a top priority status from 30% or more of respondents.
Italy	• The same proportion of respondents selected two actions as most important (47%): improve timeliness of diagnosis and facilitate more multi-disciplinary care delivery. • However, there was no strong agreement as no single area was chosen as a top priority by over half of the respondents and this was an illustration of a heterogeneity of views. • Other options selected as a priority by fewer respondents included improving patient access to antibiotics (37%) and to monoclonal antibody therapy (34%) and addressing variation in access to FMT (32%). • No respondents from Italy saw improving mental health support for CDI patients as a priority.
UK	• There was a strong agreement on improving multi-disciplinary care delivery as a top priority (68%) but a variety of views on other priorities, with 44% of respondents seeing addressing variation in FMT as top priority and 40% seeing improving the timeliness of diagnosis as top priority. • No other option had 30% or more of respondents selecting it as a top priority.
France	• The same proportion of respondents selected two actions as most important (50% for both, although this is only 2 respondents): improving access to effective antibiotics and more multi-disciplinary care delivery. • In addition, 25% of respondents (1 respondent) selected improved timeliness of diagnosis as most important. One felt that none of the listed actions were priorities.

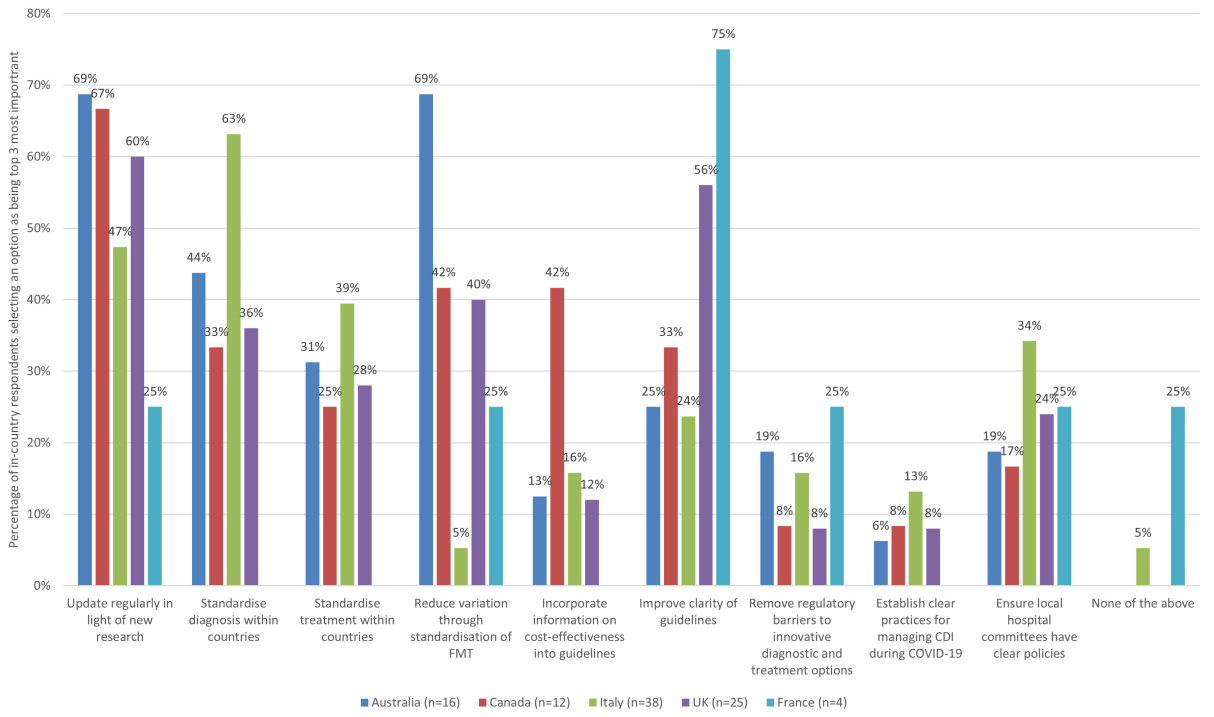

FIGURE 3
The improvement actions related to guidelines and regulation for CDI treatments that are the most important (bars represent the percentage of respondents that rated each improvement action as one of the most important by country).

improvement actions they thought were the most important (Figure 6).

Better evidence on optimal treatment regimens in managing patients with specific profiles was the most selected evidence gap across all countries (94% Australia, 83% Canada, 74% Italy, 68% UK, and 50% France saw it as a top priority). Half of respondents from Australia and Canada saw a need to address gaps and improve evidence on the efficacy and safety of emerging preventatives as a priority, but this was not the case for the Italy (37%), UK (36%), or France (0%). Half of respondents from France, and nearly half from Italy (45%) also identified a need for better evidence on risk factors associated with recurrence as a top evidence gap to address whereas this was not the case for other countries (44% Australia, 32% UK, and 17% Canada).

TABLE 6 Country-level insights relating to guideline and regulation priorities.

Country	Insights
Australia	• Strong agreement on the importance of updating diagnosis and treatment guidelines more regularly in light of new research and also saw guidelines to reduce unwarranted variation through standardization of FMT practice as a priority (69%). • However, there was a variety of views on priority areas, with 44% of respondents also noting standardizing diagnosis practices within the country as a top priority and standardizing treatment guidelines within countries was seen as a priority by 31% of respondents. • Other improvement options were seen as important by fewer than 30% of respondents.
Canada	• Strong agreement on only one option – updating diagnosis and treatment guidelines regularly in light of new research (67% selected this as a top priority). • There was a variety of views on other priority areas, with 42% seeing reducing unwarranted variation through standardization of FMT practice and incorporating information on cost effectiveness into guidelines as most important areas where improvement is needed. 33% saw standardizing diagnosis guidelines within countries and improving clarity of guidelines as most important. • Other improvement options were seen as important by less than 30% of respondents.
Italy	• Strong agreement about the importance of standardizing diagnosis guidelines within countries (63% of survey respondents selected this to be an important option). • However, there is a variety of views on the importance of other improvement opportunities. For example, 47% of respondents from Italy saw updating guidelines regularly as a top priority, 39% saw standardizing treatment guidelines as key and 34% saw ensuring local hospital committees have clear policies as most important. Other options were seen as important by less 30% of participants.
UK	• Strong agreement about the importance of updating guidelines in light of new research (60% of respondents) and improving clarity of guidelines (56%). • No other area was seen as a top priority by half or more of survey respondents, but 40% saw reducing unwarranted variation through standardization of FMT practice as a top priority. Less frequently selected as key was standardizing diagnostic guidelines (36%). • Other improvement options were seen as a top priority by less than 30% of respondents, but do illustrate the heterogeneity of views.
France	• Three-quarters of respondents selected improving clarity of guidelines as a top priority. • Other improvement options were seen as a top priority by less than 30% of respondents and no respondents from France selected the other options.

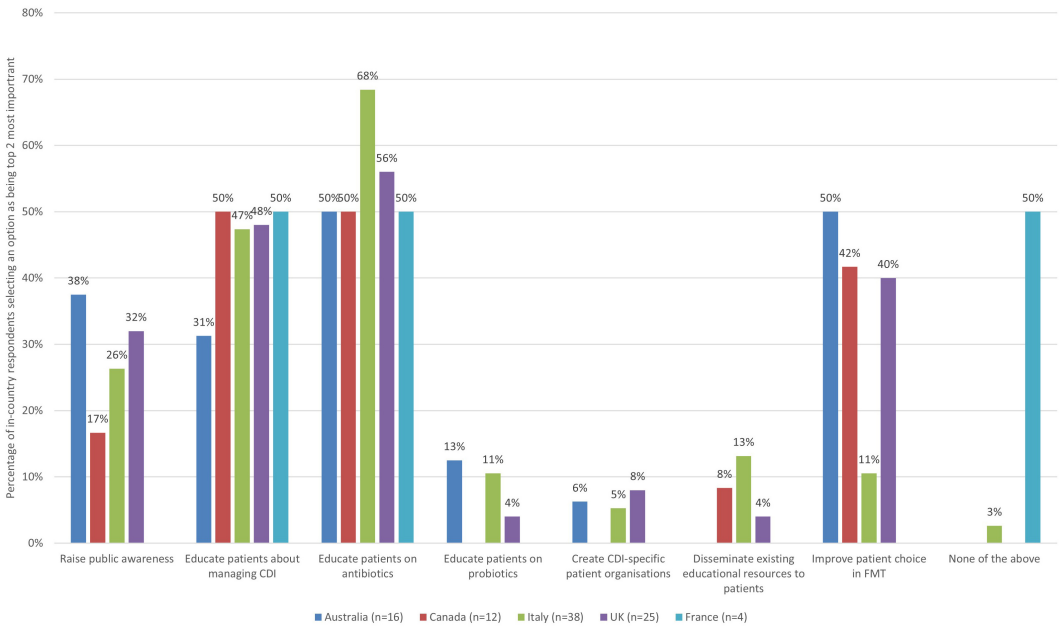

FIGURE 4
The 2 improvement actions related to education and awareness raising for patients that are the most important (bars represent the percentage of respondents that rated each improvement action as one of the most important by country).

When zooming into country level data, some notable findings are presented in Table 9.

3.4.7. Other improvement opportunities and evidence gaps

In the survey, respondents were also provided the opportunity to share views on any additional improvement activities and evidence gaps that had not been included in the survey already. For improvement opportunities, many responses focused on infection prevention and control (which is out of scope for this study). Some stressed evidence gaps or improvement actions that had already been included in the survey questions. Additional improvement actions that were identified included: improving diagnosis of *C. difficile* carriers where the patient has active chronic inflammation of the bowel (e.g., IBD), improving the exclusion of other (non-CDI) causes of diarrhea (e.g., other infections and laxatives) to improve antibiotic stewardship, improving methods for collecting data on stool frequency and consistency, and general improvements to antibiotic prescribing.

Respondents also shared views on some additional evidence gaps related to infection prevention and control or reinforced evidence gaps that had already been covered in the survey. Additional evidence gaps that were mentioned included: better evidence on

TABLE 7 Country-level insights relating to education and awareness raising for patients priorities.

Country	Insights
Australia	• Half of respondents saw educating patients with CDI on the appropriate use of antibiotics and improving patient choice in relation to FMT as priority actions. • In addition, 38% selected raising awareness of CDI among the public and 31% educating patients on management of CDI and potential future impacts as most important. • All other actions were seen as most important by fewer than 30%, and none selected improving dissemination of existing educational resources to patients as a top priority.
Canada	• Half of survey respondents saw two improvement areas as standing out in terms of importance, the one being educating patients with CDI on the appropriate use of antibiotics and the other educating patients with CDI about the management of the illness. • In addition, 42% saw improving patient choice in relation to FMT as a priority action. • All other actions were selected as most important by fewer than 30% of respondents from Canada. None selected educating patients on probiotics or creating CDI-specific patient organizations as top priorities.
Italy	• For Italy, more than half of respondents selected educating patients on appropriate use of antibiotics as the top priority (68%). • Nearly half (47%) selected educating patients with CDI about the management of the illness as a top priority. • All other actions were selected as most important by fewer than 30% of respondents.
UK	• More than half of respondents saw the need for educating patients on appropriate use antibiotics as the top priority (56%). • Some other priorities, though not with strong agreement across respondents were educating patients with CDI about the management of the illness (48%), improving patient choice in relation to FMT (40%) and raising awareness of CDI among the public (32%) as top priorities. • All other actions were selected as most important by fewer than 30%.
France	• The same proportion of respondents saw two actions as most important (50% for both, 2 respondents): educating patients with CDI on the appropriate use of antibiotics and educating patients with CDI about the management of the illness. • Half of respondents selected 'none of the above.' • No respondents from France selected that any of the other improvement actions were most important.

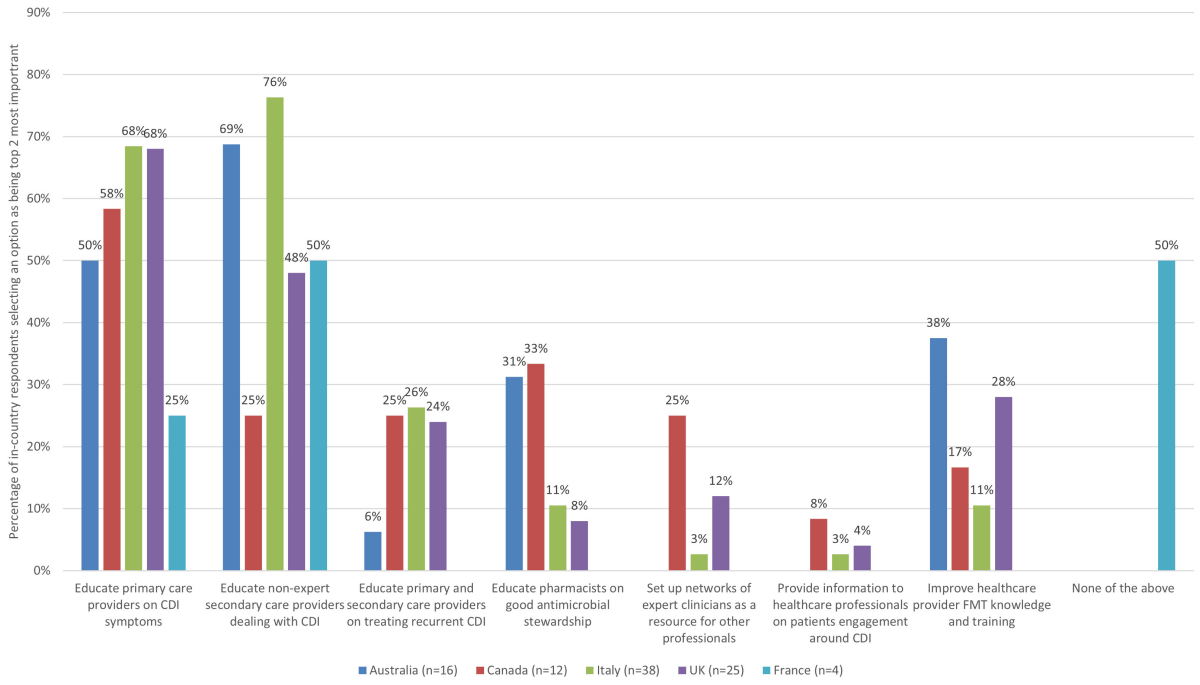

FIGURE 5
The 2 improvement actions related to education and awareness raising for clinicians that are the most important (bars represent the percentage of respondents that rated each improvement action as one of the most important by country).

particular treatment regimes (e.g., for the first CDI episode to prevent recurrences), co-managing CDI and IBD, making a reliable diagnosis (e.g., interpreting test results and identifying cure), documenting the biological mechanisms of CDI, providing treatment in cases of positive test results but no clinical symptoms, involvement of community pharmacists alongside primary care, FMT (e.g., for first episode CDI and using synthetic material), and research into the prevention of recurrences.

4. Discussion

4.1. Reflecting on improvement priorities and future research needs

This paper contributes to understanding key challenges and areas of need of improvement in the care of patients with CDI, as they relate to the clinical care pathway and the wider healthcare system which

TABLE 8 Country-level insights relating to education and awareness raising of clinician's priorities.

Country	Insights
Australia	• There was clear consensus on the top two priorities, selected by at least half of respondents. These were educating non-expert secondary care providers on dealing with CDI (69% selected as most important) and educating and supporting healthcare professionals in primary care (50%). • In addition, 38% saw improving healthcare provider FMT knowledge and training as most important, and 31% saw educating pharmacists on good antimicrobial stewardship as most important. • All other actions were selected by fewer than 30% of respondents, and no respondents from Australia saw setting up networks of experts providing information and educational support for healthcare professionals on how to effectively and confidently engage with patients as priority actions.
Canada	• Only one action was selected as most important by more than half of respondents: educating and supporting healthcare professionals in primary care (58%). • In addition, 33% saw educating pharmacists on good antimicrobial stewardship as most important. • All other actions were selected by fewer than 30% of respondents.
Italy	• There was clear consensus on the top two priorities, selected by over half of respondents. These were educating non-expert secondary care providers on dealing with CDI (76%) and educating and supporting healthcare professionals in primary care (68%). • All other actions were selected as most important by fewer than 30% of respondents.
UK	• Over half of respondents (68%) saw educating and supporting healthcare professionals in primary care as most important. • This was followed by 48% selecting educating non-expert secondary care providers on dealing with CDI as most important. • All other actions were selected as most important by fewer than 30%.
France	• For France, half of respondents (2 respondents) selected educating non-expert secondary care providers on dealing with CDI as most important. • In addition, 50% also selected 'none of the above.' • All other actions were selected as most important by fewer than 30%, with no respondents selecting four of the options as important.

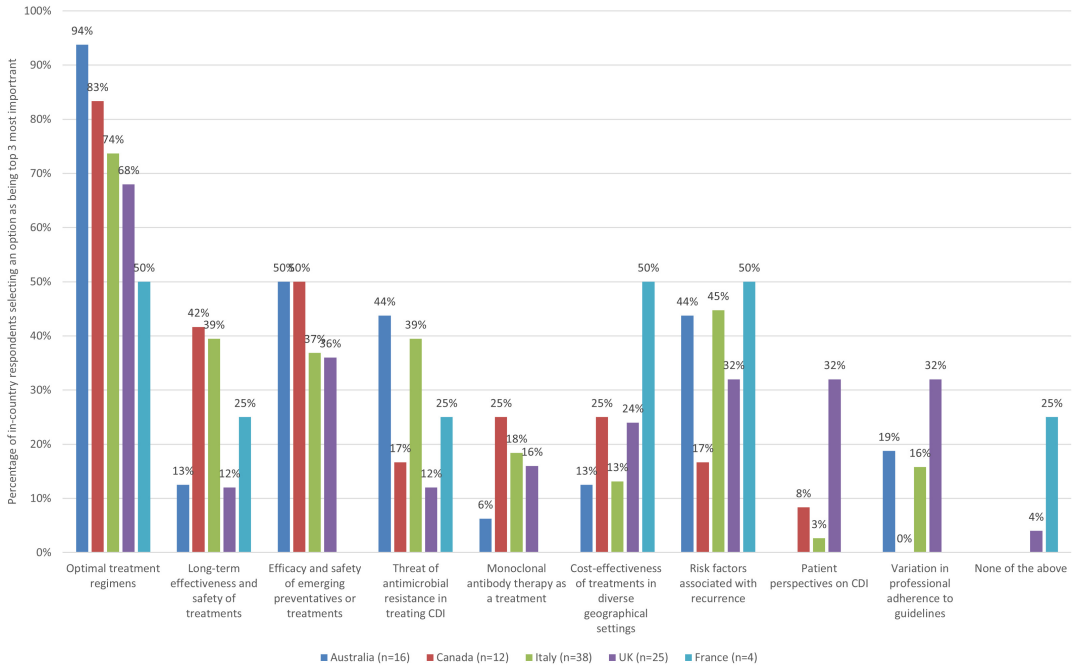

FIGURE 6
The 3 evidence gaps in relation to CDI that are the most important to address by country.

frames its operations. In doing so it contributes to the knowledge base on how patient care could be optimized, considering similarities and differences in a sample of high-income country contexts (i.e., case example countries), and in light of the wider literature that covers a broader set of geographies and contexts.

When examining the findings, it is striking that there are both similarities and differences in priority areas for improvement in different contexts. However, what is recognized across the different examined geographies is the need for improvement actions targeting *both* innovation for clinical care directly (e.g., developing innovative treatments) and those targeting the way healthcare systems enable high quality care (e.g., through keeping guidelines up to date, education and awareness raising efforts, and health system organization).

In the following, we discuss lessons learned from the stakeholder survey and how they relate to broader ideas about challenges from the literature, expert interviews and workshops.

In doing so we focus on areas of agreement but recognize that there are also improvement actions where there was less consensus, but which are still important to segments of the populations involved in patient care.

When considering survey insights on improvement needs related to the clinical care pathway, we observed high levels of agreement on the need to develop innovative products for *preventing* recurrence

TABLE 9 Country-level insights relating to evidence gaps priorities.

Country	Insights
Australia	• One evidence gap was selected by nearly all respondents (94%): better evidence on optimal treatment regimens in managing patients with specific profiles. • There was also strong agreement on another: 50% saw better evidence on efficacy and safety of emerging CDI preventatives/treatments as most important to address. • In addition, 44% of respondents from Australia thought both better evidence on the threat of AMR in the treatment of CDI and better evidence on risk factors associated with recurrence were most important. • All other evidence gaps were selected by fewer than 30% of respondents. No respondents from Australia saw better evidence on the patient perspective of CDI as most important to address.
Canada	• The majority of respondents (83%) saw better evidence on optimal treatment regimens in managing patients with specific profiles as the most important evidence gap. • Half saw better evidence on efficacy and safety of emerging CDI preventatives/treatments as most important and 42% saw better evidence on long-term effectiveness/safety of treatments key to address. • All other evidence gaps were selected by fewer than 30% of respondents. No respondents from Canada saw better evidence on variation in professional adherence to guidelines as most important to address.
Italy	• Nearly three-quarters of respondents (74%) felt that better evidence on optimal treatment regimens in managing patients with specific profiles was the most important evidence gap to tackle. • In addition, 45% selected better evidence on risk factors associated with recurrence and 39% selected both better evidence on long-term effectiveness/safety of treatments and better evidence on the threat of AMR as most important. • All other evidence gaps were selected by fewer than 30% of respondents.
UK	• 68% of respondents better evidence on optimal treatment regimens in managing patients with specific profiles as the most important evidence gap. • In addition, 36% selected better evidence on efficacy and safety of emerging CDI preventatives/treatments as most important. Nearly one-third (32%) selected three evidence gaps as most important: better evidence on risk factors associated with recurrence, on the patient perspective of CDI and on variation in professional adherence to guidelines. • All other evidence gaps were selected by fewer than 30% of respondents.
France	• The same proportion of respondents selected three gaps as most important (50% for all, however, this only represents 2 respondents): better evidence on optimal treatment regimens in managing patients with specific profiles, on the cost-effectiveness of treatments in diverse geographical settings and risk factors associated with recurrence. • All other evidence gaps were selected by fewer than 30% of respondents. No respondents from France selected better evidence on efficacy and safety of emerging CDI preventatives/treatments, on monoclonal antibody therapy as a CDI treatment, patient perspective on CDI or variation in professional adherence to guidelines as most important.

across all surveyed countries and, in most case example countries, developing innovative and more effective *treatments* for recurrent CDI was also seen as a priority (Italy, Australia, Canada and, for prevention only, the UK). This resonates with insights from the literature (as reported on earlier in this paper) flagging higher demands on healthcare services in terms of managing and dealing with recurrence. In addition, in the UK there was strong agreement on the need for developing new treatments for initial CDI. This may be due to the UK having had performance management in place for decades for the management of CDI (e.g., targets and objectives) and so experts may be sensitized to the need to improve treatment options. In light of wider treatment challenges for initial CDI identified in the analyzed literature and stakeholder consultation, further research is needed to understand whether key improvements are needed in clinical effectiveness, cost-effectiveness or reduction of side-effects.

When exploring insights related to patient monitoring, the reviewed literature and stakeholder consultation identified challenges with knowing when a patient is cured, which could have implications on patient treatment decisions and healthcare resource utilization. This did not come up as an area of priority in terms of improvement in the survey data, but evidence suggests a need for further research on how to assess 'cure' (for example how long symptoms need to be absent before a patient is considered cured and how to accurately test if a patient is cured).

In terms of access and organization of service delivery and quality of care, areas where agreement on the need to improve was the strongest included actions to address variation in access to FMT at local, regional and national levels (Canada and Australia) and actions to facilitate more multi-disciplinary patient care (UK, Australia, and France). Improving access to FMT also resonates with findings from the conducted literature review, workshops and interviews. In addition, respondents from some countries identified improvement priorities related to access to effective antibiotics for treating CDI (Canada and France) and timeliness of diagnosis (Australia and France). Views on priorities in terms of improving organization of service delivery and quality of care were particularly diverse within Italy where there was no strong agreement on any one area being most important, but with five areas being seen as priorities by a third or more of survey respondents: improving timeliness of diagnosis, facilitating more multi-disciplinary care delivery, improving patient access to antibiotics, improving access to monoclonal antibody therapy and addressing variation in access to FMT. In general, the observed similarities and variety across surveyed countries is likely to derive from specificities of healthcare system organization, capacity and infrastructure.

Our research also points to the impact of guidelines and regulation on care quality, and to the scope to improve guideline contents and the wider support that healthcare systems can provide to improve adherence. There was strong agreement amongst respondents from the majority of surveyed countries on the importance of updating diagnosis and treatment guidelines in light of new knowledge, with over half of respondents in Australia, Canada, and UK seeing this as a top priority, and nearly half in Italy. This resonates with insights obtained through international interviews and literature on European practices, which highlight outdated guidelines being a challenge to optimizing patient care. Although warranting further research, outdated guidelines may also

be linked to challenges in adherence to guidelines identified in the literature and discussed earlier. Here, it is important to note that The European Society of Clinical Microbiology and Infectious Diseases guidelines for treating CDI were updated in late 2021, but progress with the implementation of the new guidelines remains to be seen. Other factors such as economic resource constraints were also identified in the literature and in stakeholder consultation as impacting on the feasibility of adhering to some guidelines. Clinician preferences and lack of audit– as discussed in the literature and reported on earlier- may also play a role in guideline adherence. In Italy, but not other surveyed countries, there was strong agreement that standardizing diagnosis guidelines was important, perhaps due to diverse practices in terms of diagnostic testing in different parts of the country. In Australia standardization was seen as important in the context of FMT practice in particular. In the UK and France, improving guideline clarity was also seen as a priority by half or more of respondents.

Our research flagged that engaging with patients with CDI around education and awareness raising on the appropriate use of antibiotics is also important for healthcare systems to consider as part of efforts to improve patient outcomes. There was strong agreement on this across respondents in all surveyed countries. Other related priority actions where there was strong agreement amongst respondents within some countries included educating patients about the management of CDI and the potential impact of the disease on their lives, (Canada and France) and improving patient choice with respect to FMT (Australia). Importantly, the survey targeted clinical and scientific experts, and did not flag combating stigma or embarrassment as a key priority, but this is a challenge identified in other stakeholder consultation (e.g., interviews and workshops), particularly from patient representatives, and merits future consideration.

Finally, information and knowledge gaps were also identified as an area for attention in terms of future actions within healthcare systems. In most case example countries (Italy, UK, Canada, and Australia) survey respondents saw as top priority the need to educate and support primary care professionals on identifying CDI symptoms, when and how to test and diagnose patients with CDI (or refer for testing and treatment to a specialist) and how to manage patients who are being treated. Educating and supporting healthcare professionals in secondary care who are not experts regularly dealing with patients with CDI was also identified as needing attention and being a priority in some countries (Italy, Australia, and France). This resonates with the challenges identified in the analysis of the literature and stakeholder consultation, especially in the context of risks of underdiagnosis and misdiagnosis and potential challenges associated with time to diagnosis, as discussed earlier in this paper.

Our analysis also identified diverse evidence gaps which would need to be addressed to support optimal patient care. In reflecting on the insights gained, it is clear that tackling any future research agenda calls for both basic science, social science and health systems research approaches and perspectives, as both clinical and behavioral evidence gaps exist in the current knowledge base. Ambitions to improve patient care will therefore depend on the ability to orchestrate clinical practice interventions and wider behavioral and systems-level actions. It would also be important to evaluate the impact of any interventions over time, both in terms of impacts on patient health and quality of life, but also on wider society and any economic implications.

Reflecting on insights from the stakeholder survey, the need for further research on optimal treatment regimens for patients with different profiles stood out as an area where there was strong agreement on this being a priority for a future research agenda. Given the survey respondents largely represent clinical experts, this is not surprising, but it also resonates with findings from the literature review, particularly in the context of challenges with treating patients who may be elderly, frail, with complex needs or comorbidities. Research into optimal treatment regimens would need to consider both clinical and cost-effectiveness, and patient experience. In some countries, survey respondents also placed particular emphasis on improving the evidence base on preventing recurrence – such as evidence on the safety and efficacy of emerging preventatives/treatments (Australia, Canada), and in France (though only a small absolute number of respondents), better evidence on risk factors associated with recurrence and better evidence on treatment cost-effectiveness were also seen as key areas meriting more research.

However, when reflecting on the overall insights gained from the literature, interviews and workshops, it is clear that improving patient care calls for advances in research in a number of other areas as well. For example, the analysis and triangulation of the stakeholder consultation data from multiple sources (e.g., interviews, workshops, and survey) and literature suggests needs to also conduct additional research on how CDI affects patient quality of life and also the experience of carers; on how potential stigma and disgust in discussing bowel problems impacts on patients accessing care, and research into the nature of interactions between patients and healthcare professionals.

We also explored variations in practice, and these too point to avenues of relevance for a future research agenda. For example, we noted diversity in referral behaviors both within and across countries (e.g., whether a patient who presents to a community care setting is referred to gastroenterologists or infectious disease specialists, or elsewhere in the system); diversity as to where diagnostic testing takes place (e.g., in public or private labs); in the combination and order of use for diverse tests used to diagnose patients, in the choice and combination of antibiotic options used to treat patients, and in the degree of multidisciplinary care involved in monitoring patients. Some of this variation in practice may be warranted in light of patient symptoms and healthcare system organization, while other areas of variation may be more subject to personal preferences and experiences of healthcare professionals or resource and capacity constraints. Further research is needed to explore where variation may or may not be warranted. For example, our evidence suggests that the frequent use of multiple diagnostic tests has both time and cost implications and there may be scope to optimize practices through further research on optimal diagnostic algorithms for patients with different profiles (given that the use of diverse algorithms was identified as a challenge).

Finally, whereas this research is unique in adopting a multidisciplinary, clinical practice and health services research perspective on the care of patients with CDI, and in combining a narrative review covering diverse high income country contexts with in-depth case examples of five countries, further primary research is needed to complement the findings identified through the case examples with data from other countries. We hope the insights we have shared in this paper help inform future research agendas, as well as shed new light on the diverse and complementary ways in which the care of patients with *C. difficile* infection can be improved in the future.

4.2. Limitations

This study examines the CDI patient care pathway and discusses key challenges to optimizing patient care across the pathway- from diagnosis, to initial treatment, patient monitoring and management of recurrence. It also examines key priorities in the context of areas where improvement is needed and explores variation in views across and within countries. It is also novel in that is covers multi-disciplinary factors, bringing together clinical and healthcare service and systems perspectives and drawing on diverse evidence sources – narrative review, interviews, workshops and a survey.

There are, however, some limitations to note. Firstly, the narrative review did not include all possible articles on the topic of the CDI patient pathway. It was intended to be a focused review of key relevant evidence to identify key challenges and improvement opportunities, not a full systematic review, but following many core principles of a systematic review approach. While the consultations engaged key experts in the five country examples, a limited number of individuals were consulted through interviews (eight in total), but this was mitigated with wider survey-based consultation. Variation in clinical practice and service delivery across provinces and states of larger countries (e.g., Australia and Canada) was possibly not all captured through the interviews and there may be some challenges or improvement opportunities related to other regions that were not identified (or that do not apply as strongly to other regions). However, the survey with a much larger number of respondents should help mitigate this, especially as there were options for respondents to present additional improvement actions and evidence gaps, and coupled with insights from the literature. Finally, while the survey involved 95 participants from across the five example countries and captured a diversity of perspectives, engagement from participants from France in particular was low (four respondents) limiting the extent to which we could generalize findings in that context.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Ethics statement

Ethical approval was not provided for this study on human participants because it was judged to pose minimal risks to participants and not to require ethical approval. The research was conducted in accordance with the Declaration of Helsinki. It was reviewed retrospectively by the RAND Human Subjects Protection Committee and determined to be exempt under 45 CFR 46.104(d)(2)(ii), and, although exempt, the study's procedures and materials were found by the committee to be consistent with all rules laid out under 45 CFR 46 for the conduct of non-exempt human subjects' research. This study involved a literature review, interviews with clinical experts and patient representatives, and a survey of clinical experts. All participants gave informed consent and were provided with participant information sheets as part of this process. The patients/participants provided their written informed consent to participate in this study.

Author contributions

SM and LH were involved in the conception and design of the study. LH, SM, SS, and RR were involved in identifying the sample for data collection, with inputs from PG, TS, JD, NP, and MW. All authors contributed to data collection, analysis, and drafting of the manuscript. All authors approved the contents for publication.

Funding

This work was supported by Ferring Pharmaceuticals and delivered by the co-authors through fully independent research and analysis. SM, LH, RR, MC, and SS also received funding from Ferring Pharmaceuticals for another independent study on challenges and improvement opportunities in delivering fecal microbiota transplantation for recurrent *C. difficile* infections.

Acknowledgments

We are grateful to the interviewees and survey participants for sharing their knowledge and insights with the research team. We also appreciate the ongoing support of Teodor Burtea (MD) and Simbarashe Magwenzi of Global Medical Affairs at Ferring Pharmaceuticals, as well as Soraya Aouad and Philip Li (formerly of Ferring Pharmaceuticals) throughout the studies design and conduct. The work was conducted and analyzed in a fully independent way by the research team.

Conflict of interest

NP received fees as speaker and scientific board member from Pfizer, Tillotts, MSD, GSK, Becton & Dickinson, and ImmuneMed. MW has received consulting fees from AiCuris, Bayer, Crestone, Da Volterra, Deinove, EnteroBiotix, The European Tissue Symposium, Ferring Pharmaceuticals, GSK, Menarini, Merck, Nestlé, Paion, Paratek, Pfizer, Phico Therapeutics, Qpex Biopharma, Seres, Surface Skins, Summit, Tillotts, Vaxxilon/Idorsia, and Vedanta; lecture fees from GSK, Merck, Pfizer, Seres, and Tillotts; and grant support from Almirall, Da Volterra, EnteroBiotix, GSK, Merck, MicroPharm, Nabriva, Paratek, Pfizer, Seres, Summit, The European Tissue Symposium, and Tillotts. He is an author of: 2010, 2017, and 2021 IDSA CDI guidelines, 2013 Public Health England CDI guidelines, 2014, 2016 and 2021 ESCMID (treatment and diagnosis) CDI guidelines. He was an expert witness to the 2021 UK National Institute for Clinical Excellence (NICE) CDI treatment guideline. PG Speaker Honoraria includes Seqirus, Novartis, Gilead, Sanofi, and Janssen. His Medical Advisory Board Memberships include AstraZeneca, GSK, MSD, and Pfizer. TS has received funding from Seres, Rebiotix, Merck, and Nubiyota for studies related to *C. difficile.*

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.1033417/full#supplementary-material

References

1. Patil RL. Proton pump inhibitors and *Clostridium difficile* infection: are we propagating an already rapidly growing healthcare problem? *Gastroenterol Res.* (2013) 6:171–3. doi: 10.4021/gr575w

2. Sartelli M, Di Bella S, McFarland L, Khanna S, Furuya-Kanamori L, Abuzeid N, et al. 2019 update of the WSES guidelines for management of Clostridioides (Clostridium) difficile infection in surgical patients. *World J Emerg Surg.* (2019) 14:8. doi: 10.1186/s13017-019-0228-3

3. National Health Service [NHS],. *Clostridium difficile*. (2018). Available online at: https://www.nhs.uk/conditions/c-difficile/ (accessed January 3, 2023).

4. Guery B, Galperine T, Barbut F. Clostridioides difficile: diagnosis and treatments. *BMJ.* (2019) 366:l4609. doi: 10.1136/bmj.l4609

5. European Centre for Disease Prevention and Control. *Clostridium difficile Infections. Annual Epidemiological Report for 2016*. Stockholm: ECDC (2018).

6. Balsells E, Shi T, Leese C, Lyell I, Burrows J, Wiuff C, et al. Global burden of Clostridium difficile infections: a systematic review and meta-analysis. *J Glob Health.* (2019) 9:010407. doi: 10.7189/jogh.09.010407

7. Czepiel J, Krutova M, Mizrahi A, Khanafer N, Enoch D, Patyi M, et al. Mortality following Clostridioides difficile infection in europe: a retrospective multicenter case-control study. *Antibiotics.* (2021) 10:299. doi: 10.3390/antibiotics10030299

8. UK Health Security Agency. *Thirty-Day All-Cause Mortality Following MRSA, MSSA and Gram-Negative Bacteraemia and C. difficile Infections, 2020 to 2021*. London: UK Health Security Agency (2021).

9. Dubberke E, Olsen M. Burden of Clostridium difficile on the healthcare system. *Clin Infect Dis.* (2012) 1:S88–92. doi: 10.1093/cid/cis335

10. Ghantoji S, Sail K, Lairson D, DuPont H, Garey K. Economic healthcare costs of Clostridium difficile infection: a systematic review. *J Hosp Infect.* (2010) 74:309–18. doi: 10.1016/j.jhin.2009.10.016

11. Mitchell B, Gardner A. Mortality and Clostridium difficile infection: a review. *Antimicrob Resist Infect Control.* (2012) 1:20. doi: 10.1186/2047-2994-1-20

12. Gupta A, Ananthakrishnan A. Economic burden and cost-effectiveness of therapies for Clostridiodes difficile infection: a narrative review. *Ther Adv Gastroenterol.* (2021) 14:17562848211018654. doi: 10.1177/17562848211018654

13. Czepiel J, Dróżdż M, Pituch H, Kuijper E, Perucki W, Mielimonka A, et al. Clostridium difficile infection: review. *Eur J Clin Microbiol Infect Dis.* (2019) 38:1211–21. doi: 10.1007/s10096-019-03539-6

14. Varker T, Forbes D, Dell L, Weston A, Merlin T, Hodson S, et al. Rapid evidence assessment: increasing the transparency of an emerging methodology. *J Eval Clin Pract.* (2015) 21:1199–204. doi: 10.1111/jep.12405

15. Bowling A. *Research Methods in Health: Investigating Health and Health Services*. Maidenhead: Open University Press (1997).

16. Gnocchi M, Gagliardi M, Gismondi P, Gaiani F, De' Angelis G, Esposito S. Updated management guidelines for Clostridioides difficile in paediatrics. *Pathogens.* (2020) 9:291. doi: 10.3390/pathogens9040291

17. Borali E, De Giacomo C. Clostridium Difficile infection in children: a review. *J Pediatr Gastroenterol Nutr.* (2016) 63:e130–40. doi: 10.1097/MPG.0000000000001264

18. Kampouri E, Croxatto A, Prod'hom G, Guery B. Clostridioides difficile infection, still a long way to go. *J Clin Med.* (2021) 10:389. doi: 10.3390/jcm10030389

19. Benoist H, de La Blanchardière A, Flatres A, Isnard C, Thibon P, Saint-Lorant G. Effect of a prospective audit with intervention and feedback on Clostridioides difficile infection management. *Int J Clin Pharm.* (2020) 42:923–30. doi: 10.1007/s11096-020-01050-z

20. Crobach M, Baktash A, Duszenko N, Kuijper E. Diagnostic guidance for C. difficile infections. *Adv Exp Med Biol.* (2018) 1050:27–44. doi: 10.1007/978-3-319-72799-8_3

21. Carroll K, Mizusawa M. Laboratory tests for the diagnosis of Clostridium difficile. *Clin Colon Rectal Surg.* (2020) 33:73–81. doi: 10.1055/s-0039-3400476

22. Cataldo M, Granata G, D'Arezzo S, Tonziello G, Vulcano A, De Giuli C, et al. Hospitalized patients with diarrhea: rate of Clostridioides difficile infection underdiagnosis and drivers of clinical suspicion. *Anaerobe.* (2021) 70:102380. doi: 10.1016/j.anaerobe.2021.102380

23. Khanna S. Microbiota restoration for recurrent Clostridioides difficile: getting one step closer every day! *J Intern Med.* (2021) 290:294–309. doi: 10.1111/joim.13290

24. Denton A, Topping A, Humphreys P. Managing Clostridium difficile infection in hospitalised patients. *Nurs Stand.* (2014) 28:37–43. doi: 10.7748/ns2014.04.28.33.37.e8461

25. Davies K, Longshaw C, Davis G, Bouza E, Barbut F, Barna Z, et al. Underdiagnosis of Clostridium difficile across Europe: the European, multicentre, prospective, biannual, point-prevalence study of Clostridium difficile infection in hospitalised patients with diarrhoea (EUCLID). *Lancet Infect Dis.* (2014) 14:1208–19. doi: 10.1016/S1473-3099(14)70991-0

26. Davies K, Davis G, Barbut F, Eckert C, Petrosillo N, Wilcox M. Variability in testing policies and impact on reported Clostridium difficile infection rates: results from the pilot longitudinal European Clostridium difficile Infection Diagnosis surveillance study (LuCID). *Eur J Clin Microbiol Infect Dis.* (2016) 35:1949–56. doi: 10.1007/s10096-016-2746-1

27. Polage C, Gyorke C, Kennedy M, Leslie J, Chin D, Wang S, et al. Overdiagnosis of Clostridium difficile infection in the molecular test era. *JAMA Intern Med.* (2015) 175:1792–801. doi: 10.1001/jamainternmed.2015.4114

28. Fang F, Polage C, Wilcox M. Point-counterpoint: what is the optimal approach for detection of Clostridium difficile Infection? *J Clin Microbiol.* (2017) 55:670–80. doi: 10.1128/JCM.02463-16

29. Vent-Schmidt J, Attara G, Lisko D, Steiner T. Patient experiences with Clostridioides difficile infection: results of a canada-wide survey. *Patient Prefer Adherence.* (2020) 14:33–43. doi: 10.2147/PPA.S229539

30. Turner D, Thorburn S, Crowe A, Jardine D, Timmins C. Trends in Clostridioides difficile diagnosis before and after a change in testing algorithm. *J Microbiol Methods.* (2021) 184:106189. doi: 10.1016/j.mimet.2021.106189

31. Public Health England. *Clostridioides difficile Infection: Guidance on Management and Treatment*. London: UK Health Security Agency (2013).

32. Penit A, Bemer P, Besson J, Cazet L, Bourigault C, Juvin M, et al. Community-acquired Clostridium difficile infections. *Med Mal Infect.* (2016) 46:131–9. doi: 10.1016/j.medmal.2016.01.007

33. Oksi J, Anttila V, Mattila E. Treatment of Clostridioides (Clostridium) difficile infection. *Ann Med.* (2020) 52:12–20. doi: 10.1080/07853890.2019.1701703

34. Fitzpatrick F, Skally M, Brady M, Burns K, Rooney C, Wilcox M. European practice for CDI treatment. *Adv Exp Med Biol.* (2018) 1050:117–35. doi: 10.1007/978-3-319-72799-8_8

35. Loo V, Davis I, Embil J, Evans G, Hota S, Lee C, et al. Association of Medical Microbiology and Infectious Disease Canada treatment practice guidelines for Clostridium difficile infection. *J Assoc Med Microbiol Infect Dis Can.* (2018) 3:71–92. doi: 10.1093/cid/cix1085

36. Roshan N, Hammer K, Riley T. Non-conventional antimicrobial and alternative therapies for the treatment of Clostridium difficile infection. *Anaerobe.* (2018) 49:103–11. doi: 10.1016/j.anaerobe.2018.01.003

37. Mendo-Lopez R, Villafuerte-Gálvez J, White N, Mahoney M, Kelly C, Alonso C. Recent developments in the management of recurrent Clostridioides difficile infection. *Anaerobe.* (2020) 62:102108. doi: 10.1016/j.anaerobe.2019.102108

38. van Prehn J, Reigadas E, Vogelzang E, Bouza E, Hristea A, Guery B, et al. European society of clinical microbiology and infectious diseases: 2021 update on the treatment guidance document for Clostridioides difficile infection in adults. *Clin Microbiol Infect.* (2021) 27:S1–21. doi: 10.1016/j.cmi.2021.09.038

39. Lauda-Maillen M, Liuu E, Catroux M, Caupenne A, Priner M, Cazenave-Roblot F, et al. Treatment compliance with European guidelines and prognosis of Clostridium difficile infection according to age. *Med Mal Infect.* (2019) 49:173–9. doi: 10.1016/j.medmal.2018.08.001

40. Trubiano J, Cheng A, Korman T, Roder C, Campbell A, May M, et al. Australasian society of infectious diseases updated guidelines for the management of Clostridium difficile infection in adults and children in Australia and New Zealand. *Intern Med J.* (2016) 46:479–93. doi: 10.1111/imj.13027

41. Sridharan K, Sivaramakrishnan G. Which antimicrobial agent is likely to be the best for treating Clostridium difficile infections? A Bayesian network meta-analysis of randomized clinical trials. *Drug Res.* (2019) 69:194–200. doi: 10.1055/a-0645-1169

42. The National Institute for Health and Care Excellence. *Clostridioides difficile Infection: Antimicrobial Prescribing*. (2021). Available online at: https://www.nice.org.uk/guidance/ng199/chapter/Recommendations#treating-suspected-or-confirmed-c-difficile-infection (accessed August 19, 2022).

43. Thabit A, Alsolami M, Baghlaf N, Alsharekh R, Almazmumi H, Alselami A, et al. Comparison of three current Clostridioides difficile infection guidelines: IDSA/SHEA, ESCMID, and ACG guidelines. *Infection.* (2019) 47:899–909. doi: 10.1007/s15010-019-01348-9

44. Madoff S, Urquiaga M, Alonso C, Kelly C. Prevention of recurrent Clostridioides difficile infection: a systematic review of randomized controlled trials. *Anaerobe.* (2020) 61:102098. doi: 10.1016/j.anaerobe.2019.102098

45. Cobo J. A comprehensive approach for the patient with Clostridium difficile infection. *Rev Esp Quimioter.* (2018) 31 Suppl. 1(Suppl. 1):27–31.

46. Heimann S, Cruz Aguilar M, Mellinghof S, Vehreschild M. Economic burden and cost-effective management of Clostridium difficile infections. *Med Mal Infect.* (2018) 48:23–9. doi: 10.1016/j.medmal.2017.10.010

47. Tresman R, Goldenberg S. Healthcare resource use and attributable cost of Clostridium difficile infection: a micro-costing analysis comparing first and recurrent episodes. *J Antimicrob Chemother.* (2018) 73:2851–5. doi: 10.1093/jac/dky250

48. Hammeken L, Baunwall S, Hvas C, Ehlers L. Health economic evaluations comparing faecal microbiota transplantation with antibiotics for treatment of recurrent Clostridioides difficile infection: a systematic review. *Health Econ Rev.* (2021) 11:3. doi: 10.1186/s13561-021-00301-7

49. Asensio A, Di Bella S, Lo Vecchio A, Grau S, Hart W, Isidoro B, et al. The impact of Clostridium difficile infection on resource use and costs in hospitals in Spain and Italy: a matched cohort study. *Int J Infect Dis.* (2015) 36:31–8. doi: 10.1016/j.ijid.2015.05.013

50. UK Health Security Agency. *Quarterly Epidemiological Commentary: Mandatory MRSA, MSSA and Gram-Negative Bacteraemia, and C. difficile Infection Data (up to January to March 2022)*. (2022). Available online at: https://www.gov.uk/government/statistics/mrsa-mssa-gram-negative-bacteraemia-and-cdi-quarterly-report/quarterly-epidemiological-commentary-mandatory-mrsa-mssa-and-gram-negative-bacteraemia-and-c-difficile-infection-data-up-to-january-to-march-2022#fig6a (accessed August 19, 2022).

frontiers | Frontiers in Public Health

TYPE Mini Review
PUBLISHED 27 January 2023
DOI 10.3389/fpubh.2023.1103518

OPEN ACCESS

EDITED BY
Amir Abdoli,
Jahrom University of Medical Sciences, Iran

REVIEWED BY
Vahid Rahmanian,
Jahrom University of Medical Sciences, Iran

*CORRESPONDENCE
Ashraful Islam Khan
ashrafk@icddrb.org

SPECIALTY SECTION
This article was submitted to Infectious Diseases: Epidemiology and Prevention, a section of the journal Frontiers in Public Health

RECEIVED 20 November 2022
ACCEPTED 06 January 2023
PUBLISHED 27 January 2023

CITATION
Khan AI, Islam MT, Amin MA, Khan ZH and Qadri F (2023) Outbreak of diarrheal diseases causes mortality in different geographical locations of Bangladesh during the 2021 COVID-19 era.
Front. Public Health 11:1103518.
doi: 10.3389/fpubh.2023.1103518

Outbreak of diarrheal diseases causes mortality in different geographical locations of Bangladesh during the 2021 COVID-19 era

Ashraful Islam Khan[1]*, Md. Taufiqul Islam[1,2], Mohammad Ashraful Amin[1], Zahid Hasan Khan[1] and Firdausi Qadri[1]

[1]International Centre for Diarrhoeal Disease Research, icddr, b, Dhaka, Bangladesh, [2]School of Medical Science, Griffith University, Gold Coast, QLD, Australia

Objectives: Diarrhea is a major public health problem in low- and middle-income countries, including Bangladesh. Of the different spectrums of diarrheal diseases, cholera occurs every year, causing outbreaks and epidemics following a biannual seasonal pattern. Due to the COVID-19 pandemic, hospitalization for diarrheal diseases decreased in 2020 compared to the previous years. However, in 2021, massive outbreaks occurred in different geographical locations of the country. We described that an outbreak of diarrheal diseases causes mortality in different geographical locations in Bangladesh.

Method: In this study, we present a report of diarrhea outbreaks that were reported in 2018–2021 in different parts of Bangladesh, and data have been captured from different sources such as print and electronic media as well as from a nationwide surveillance system.

Results: Among these locations, districts of Barisal Division, Kishorganj, Noakhali, Gopalganj, Bandarban, and Chattogram were the major hotspots of the outbreaks where high morbidity due to acute watery diarrhea and even mortality, which is usually low in Bangladesh, were recorded.

Conclusion: Early detection and prevention and strengthening of the surveillance system are needed to combat the diarrheal upsurge, take immediate control, and adopt preventive strategies.

KEYWORDS

Bangladesh, cholera, diarrhea, outbreak, COVID-19

Introduction

Diarrhea is a disease of the gastrointestinal tract characterized by frequent, loose, and watery bowel movements. The etiology of the disease may be bacterial (*Vibrio cholerae*, ETEC, *Shigella*, and *Salmonella* spp.), viral (most commonly rotavirus), protozoa, and parasitic organisms, which can be spread by contaminated water (1). Diarrheal diseases have been considered a major public health problem and are estimated as the eighth leading cause of mortality globally (2). Most burden estimations have been focused on children due to their high prevalence in under five children (1.7 billion episodes annually among under five children) even though a substantial burden is seen in adults (2, 3). Overall deaths due to diarrhea have been reduced after the invention of oral rehydration solution (ORS), but morbidity has remained relatively constant (4). In Bangladesh, diarrheal diseases are the most common cause for seeking hospital-level

care (5, 6). Along with over 210 countries globally, Bangladesh is also facing a large outbreak of COVID-19 at present. The World Health Organization (WHO) has declared it a pandemic emergency, and the first-ever COVID-19 case was detected in Bangladesh on 8 March 2020 (7). Due to the COVID-19 pandemic, hospitalization for diarrheal diseases decreased in 2020 compared to that in previous years. However, in 2021, massive outbreaks occurred in different geographical locations of the country. Epidemics and outbreaks are common in the region where there is a shortage of clean water for drinking, cooking, and cleaning and also among people with a lack of knowledge on basic hygiene and sanitation. Most importantly, water contaminated with feces from municipal sewage, septic tanks, and latrines is the cause of disease outbreaks. However, water scarcity, increased salinity of water, and climate change are the predisposing factors for increased diarrheal disease outbreaks. Monitoring outbreaks can help us learn more about the causes of outbreaks, sources, and the groups of people who become ill. This knowledge can be used to control the outbreak and prevent the further spread or recurrence of the infection in future. Diarrheal diseases including cholera outbreaks occurred in Bangladesh many times, including during floods (1988, 1998, and 2004) and due to behavioral factors such as using contaminated sources of water and poor hygiene practices (8, 9). Cholera is an extremely virulent disease that can cause severe acute watery diarrhea, and the WHO has launched an aim to end cholera by 2030, with the major target being to eliminate cholera from 20 countries out of 47 cholera-endemic countries by 2030 (10). To align with this target, burden estimation, following the disease trends, integrated disease surveillance, and rapid outbreak investigation are crucial. The reason behind this may have been the fear of seeking treatment in hospitals, maintaining good hand-washing practices, and avoiding street food due to the COVID-19 pandemic. However, from April 2021, the country experienced sporadic outbreaks of diarrheal diseases in different districts, causing a huge number of cases and deaths. In this report, a brief update on this upsurge/outbreak of diarrheal diseases including cholera is described based on reports from different media.

Method/data source

Information on diarrheal outbreaks was collected from different print and electronic media as well as personal communication with different hospitals and institutes around the country. We adopted various electronic data sources for this report, which include newspaper and television news broadcasts. Very limited data on the microbiological cause of outbreaks were available. However, nationwide sentinel cholera surveillance has been continuing in 16 sites in Bangladesh in collaboration between icddr,b and the Institute of Epidemiology Disease Control and Research (IEDCR) (11). In this surveillance, participants with acute watery diarrhea have been enrolled, and also a rapid diagnostic test (RDT) and microbiological culture have been carried out for collected samples. We have also used the data from this surveillance network for this outbreak report.

Result and discussion

Barisal Division, located in south-central Bangladesh, has been affected by diarrheal diseases since the beginning of this year.

TABLE 1 Number of cases and deaths due to diarrheal disease outbreaks in different districts/upazila in Bangladesh, 2021.

District	Case	Death
Bhola	9,355	00
Barisal	4,989	05
Patuakhali	6,290	03
Pirojpur	4,204	00
Barguna	5,634	02
Jhalokati	3,797	00
Bandarban/ Alikadam	136	06
Kishoreganj/Mithamoin	100	04
Gopalganj	1,563	00
Noakhali	10,000	15
Bhasan Char (Hatiya)	1,500	04
Total	**47,568**	**39**

According to one report by a local newspaper, over 50,000 patients received treatment for diarrheal diseases in health facilities between 1st January and 10th May 2021. According to government records, 19 patients have died whereas non-government sources have recorded 36 deaths (12). Between 1st April and 23rd April, a total of 38,046 cases and 10 deaths were reported, with the highest number of cases in Bhola and the highest number of deaths in the Barisal district in Bangladesh's Barisal division (Table 1). In addition, an average of more than 1,000 people was admitted to hospitals each day in mid-April 2021. Considering this critical situation, a national 'outbreak response' from the Institute of Epidemiology Disease Control and Research (IEDCR) was carried out. The groups worked in these areas to investigate the burden and etiology of these upsurges. After laboratory testing, *Vibrio cholerae* O1, *E. coli,* and other bacteria were found in the stool sample of the affected patients. It was assumed that contaminated water and other environmental factors, such as scarcity and increased salinity of water, were the sources of infection. Usually, the people in these areas used tube well for drinking water but for other household purposes such as cooking and washing, they used water from natural sources (rivers and ponds). Furthermore, people are used to eating "Panta" (cooked rice soaked in water overnight or longer), and for this preparation, they use water from natural sources. Government officials have warned the public that the magnitude of diarrhea and deaths caused by AWD diarrhea in Barisal has surpassed previous two-decade records (13, 14). The Ministry of Health and Family Welfare (MOH&FW) took immediate action to combat this epidemic by ensuring medicines and saline and by taking some action of awareness. The nationwide sentinel surveillance covers three outbreak areas (Barisal, Patuakhali, and Pirojpur). During the outbreak in April 2021, a total of 147 samples were collected from these areas. Among the tested sample, 28% ($n = 41$) was RDT positive, and also 16% ($n = 23$) of the culture-confirmed organism has been isolated from stool samples for *Vibrio cholerae* O1.

In addition to Barisal, the upsurges were sporadically reported from other eight divisions of Bangladesh including Dhaka and Chattogram. A diarrheal outbreak occurred in early May 2021 in

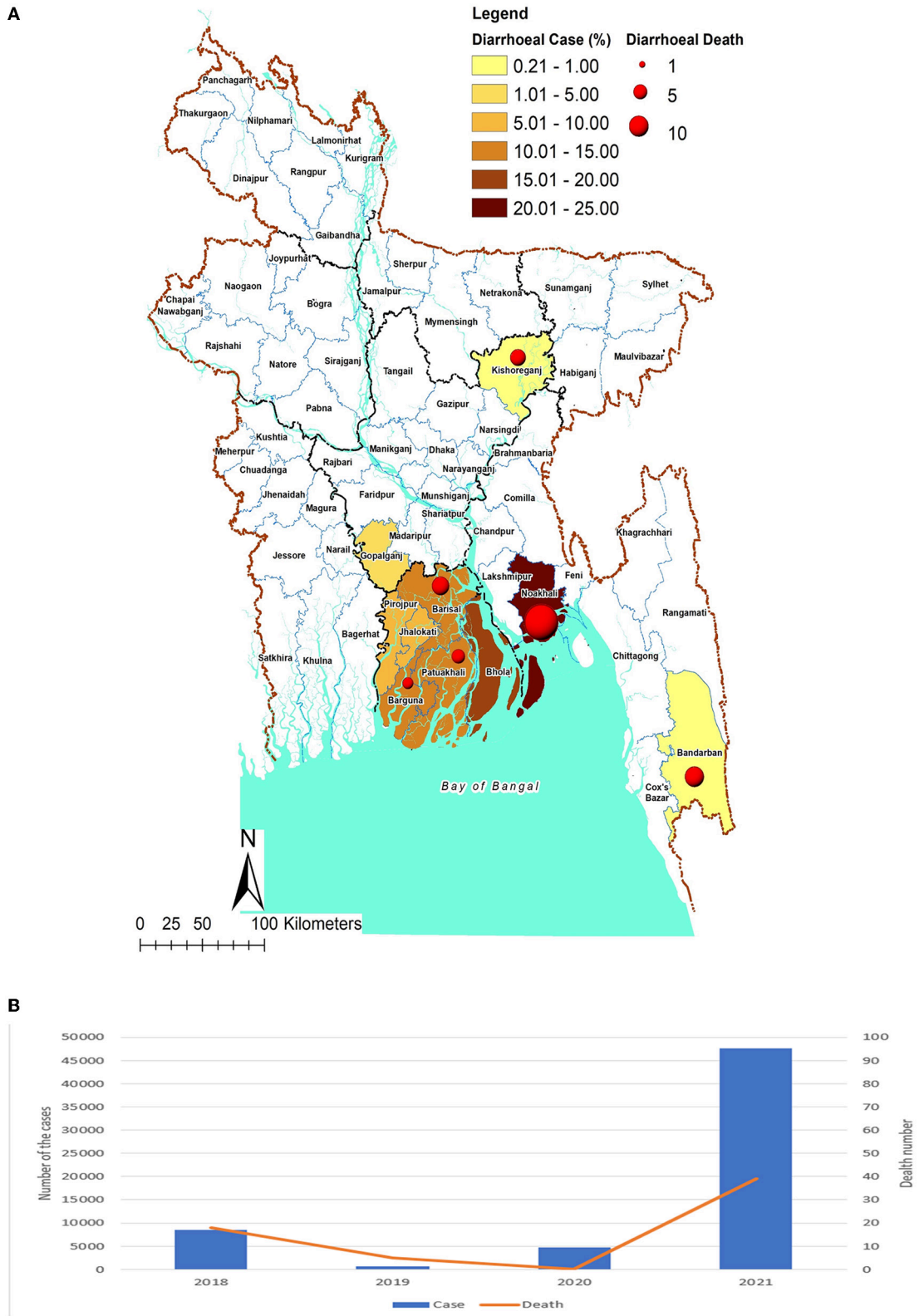

FIGURE 1
(A) The number of cases and death due to diarrhoeal discase outbreaks in different districts/upazila in Bangladesh, 2021. Map source: Banglapedia Bangladesh map digitize and data updated by icddr,b. **(B)** The number of cases and deaths dur to diarrhoeal disease outbreaks in different districts/upazlia in Bangladesh, 2018–2021; Data source from the print and electronic media.

Mithamoin upazila of Kishoreganj District in northern Bangladesh, located in the Mymensingh Division, and more than a hundred children and adults were affected by diarrhea and four individuals died as a result of severe dehydration. In Gopalganj in the Dhaka division, the diarrheal upsurge was recorded from April onward. Increased salinity and pollution of the surrounding river "Modhumoti" in the

area were considered major sources of infection (15). Noakhali, a district of Chattogram Division faced a similar outbreak in April–May 2021, where 15 deaths due to diarrheal diseases were notified in 20 days. Around 10,000 people were affected by the disease at that time and a majority of the cases were children and the elderly (16). In early June 2021, diarrhea cases also rose in Alikadam Upazila of Bandarban, and six deaths due to AWD were notified within 4 days from the affected area. The health authority of the Bandarban area and the Military patrol team worked together to control the epidemics by ensuring adequate drinking water, water purification tablets, saline, and other essential medicines (17). The Forcibly Displaced Myanmar Nationals (FDMNs) who fled to Bangladesh in 2017 due to the internal conflict in Myanmar resided in Cox's Bazar. In December 2021, approximately 20,000 FDMNs were shifted to Bhasan Char, an isolated island in Hatiya under the Noakhali district. A diarrheal epidemic was also observed in that area, where 1,500 people were infected along with four deaths. The local health authorities confirm that they were able to control the situation in Bhasan Char (18). We also provide a geographical distribution of the 2021 diarrhea outbreak in Bangladesh (Figure 1A). We have searched different reports to find out the morbidity and mortality due to diarrheal diseases across the country for the period between 2018 and 2021. The death counts and hospitalization rates were higher in 2021 in comparison to other years (19–34) (Figure 1B). Rotavirus, adenovirus, and *Vibrio cholerae* were the most common diarrheagenic microorganisms in Bangladesh, regardless of age or location. It is critical to speed up the introduction of rotavirus and cholera vaccines into the national vaccination program, as these vaccines have the potential to considerably lower the burden (35). The intensity of diarrhea is usually noticed more in places adjacent to coastal environments, particularly in Bangladesh's southern coastal areas and north-eastern regions. The northeastern territory is particularly vulnerable owing to the annual occurrence of severe flooding during the rainy season.

The recent cholera epidemics that occurred in South America (36), Asia (37), and sub-Saharan Africa (38) affected millions of people and had a high mortality rate. The World Health Organization (WHO) documented annual cholera incidences globally (39). Although these are mainly focused on official incidents that the affected countries have documented. These reports are believed to be underestimated due to limitations or lack of adequate surveillance systems. In addition, the actual global number of cholera cases may be estimated to be higher than officially reported (40). Because outbreaks are frequently not reported to avoid the risk of travel and trade embargoes against the affected country. In recent diarrheal outbreaks in Bangladesh, analysis of acute diarrhea cases showed *V. cholerae* to be the most commonly identified causative agent (41).

Primary data were not used in this study, so that was one of the primary limitations. One of the strengths was that the combined data presentation did highlight the 2021 diarrhea outbreak, including mortality, so this will create awareness about future outbreaks of diarrhea in Bangladesh.

Conclusion

Diarrheal diseases occur every year in Bangladesh, but in 2021, the cases and fatality rates exceeded previous reports in some places. *Vibrio cholerae* is usually a cause of diarrheal epidemics and outbreaks in Bangladesh (11); however, due to the lack of microbiological data, we are unable to determine the cause. The establishment of a national surveillance network with enhanced laboratory capacity for early detection and immediate action is key for combating the disease. However, to achieve the target of cholera ending by 2030, different intervention strategies such as improvement of water sanitation and hygiene facilities, immunization including cholera vaccine in the hotspot, household water treatment, and preventive treatment for household contact can play a major role in preventing diarrheal disease. In conclusion, enhanced awareness and alert systems, sustainable surveillance, and epidemiological studies can track trends in diarrheal disease incidence and mortality along with future projections, which will lead to evaluations of different prevention and control strategies.

Author contributions

The article's first draft was written by AK, MI, and MA. AK, FQ, MA, MI, and ZK contributed to the literature review and manuscript preparation. All authors contributed to the final version by critically reviewing and editing drafts.

Acknowledgments

icddr,b grateful to the governments of Bangladesh, Canada, Sweden, and the United Kingdom for providing core/unrestricted support.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Mosisa D, Aboma M, Girma T, Shibru A. Determinants of diarrheal diseases among under five children in Jimma Geneti district, Oromia Region, Ethiopia, 2020: a case-control study. *BMC Ped.* (2021) 21:1–3. doi: 10.21203/rs.3.rs-267187/v1

2. GBD 2016 Diarrhoeal Disease Collaborators. Estimates of the global, regional, and national morbidity, mortality, and aetiologies of diarrhoea in 195 countries: a systematic analysis for the global burden of disease study 2016. *Lancet Infect Dis.* (2018) 18:1211–28. doi: 10.1016/S1473-3099(18)30362-1

3. Bapanpally N, Shree GVU, Ranjeet M, SundarJunapudi S. Knowledge, attitude, and practice of mothers of under-five children regarding diarrheal illness: a cross sectional study Hyderabad. *Int J Health Clin Res.* (2021) 4:182–5.

4. Deac LM. Infectious Diarrhea, as Public Health Problem, in Romania. doi: 10.31579/2690-8794/070

5. Karim F, Khan ANS, Tasnim F, Chowdhury MAK, Billah SM, Karim T, et al. Prevalence and determinants of initiation of breastfeeding within one hour of birth: an analysis of the Bangladesh Demographic and Health Survey, 2014. *PLoS ONE.* (2019) 14:e0220224. doi: 10.1371/journal.pone.0220224

6. Sultana M, Mahumud RA, Sarker A. Emerging patterns of mortality and morbidity in district level hospitals in Bangladesh. *Ann Public Heal Res.* (2015) 2:2–4.

7. Team E. The epidemiological characteristics of an outbreak of 2019 novel coronavirus diseases (COVID-19)—China, 2020. *China CDC weekly.* (2020) 2:113. doi: 10.46234/ccdcw2020.032

8. Andrews JR, Leung DT, Ahmed S, Malek MA, Ahmed D, Begum YA, et al. Determinants of severe dehydration from diarrheal disease at hospital presentation: evidence from 22 years of admissions in Bangladesh. *PLoS Negl Trop Dis.* (2017) 11:e0005512. doi: 10.1371/journal.pntd.0005512

9. Schwartz BS, Harris JB, Khan AI, Larocque RC, Sack DA, Malek MA, et al. Diarrheal epidemics in Dhaka, Bangladesh, during three consecutive floods: 1988, 1998, and 2004. *Am J Trop Med Hyg.* (2006) 74:1067–73. doi: 10.4269/ajtmh.2006.74.1067

10. Organization WH. *Ending Cholera a Global Roadmap to 2030*. World Health Organization (2017). p. 32. Available online at: https://www.gtfcc.org/wp-content/uploads/2020/09/ending-cholera-a-global-roadmap-to-2030.pdf

11. Khan AI, Rashid MM, Islam MT, Afrad MH, Salimuzzaman M, Hegde ST, et al. Epidemiology of cholera in Bangladesh: findings from nationwide hospital-based surveillance, 2014–2018. *Clin Infect Dis.* (2020) 71:1635–42. doi: 10.1093/cid/ciz1075

12. ProthomALo. Over 50,000 diarrhoea patients in Barishal Division 2021. Available online at: https://en.prothomalo.com/bangladesh/local-news/over-50000-diarrhoea-patients-in-barishal-div-2 (accessed July 20, 2021).

13. Express TF. Outbreak of diarrhoea in Barishal Division 2021. Available online at: https://www.thefinancialexpress.com.bd/editorial/outbreak-of-diarrhoea-in-barishal-division-1619538451 (accessed July 20, 2021).

14. Star TD. Cholera germs in rivers, canals behind Barishal's diarrhoea cases: IEDCR 2021. Available online at: https://www.thedailystar.net/city/news/cholera-germs-rivers-canals-behind-barishals-diarrhoea-cases-iedcr-2082253 (accessed August 5, 2021).

15. Tribune D. Diarrhoea patients on the rise in Gopalganj 2021. Available online at: https://www.dhakatribune.com/bangladesh/nation/2021/05/19/diarrhoea-patients-on-the-rise-in-gopalganj (accessed July 26, 2021).

16. Star TD. Diarrhoea cases rising due to heat, 15 dead so far in Noakhali 2021. Available online at: https://www.thedailystar.net/health/news/diarrhoea-cases-rising-due-heat-15-dead-so-far-noakhali-2098637 (accessed August 27, 2021).

17. risingbd. 6 die of Diarrhoea in Bandarban 2021. Available online at: https://www.risingbd.com/english/country/news/80249 (accessed July 20, 2021).

18. AlJAZEERA. Diarrhoea kills at least 4 Rohingya on remote Bangladesh island. (2021) Available online at: https://www.aljazeera.com/news/2021/6/18/diarrhoea-bangladesh-rohingya-island (accessed August 27, 2021).

19. "Four thousand" affected by diarrhea in one month in Gazipur. *bdnews*. (2018) 24. Available online at: https://bangla.bdnews24.com/samagrabangladesh/article2042868.bdnews (accessed August 27, 2021).

20. Diarrhea situation in Gaibandha has worsened. *Somoyer konthosor*. (2018).

21. One child died of diarrhea in Kurigram, 250 were affected. *Prothom alo*. (2018) 24. Available online at: https://www.somoyerkonthosor.com/2018/04/01/210599.htm (accessed August 27, 2021).

22. Police officer dies of diarrhea. *Dhakatimes*. (2018) 24. Available online at: https://www.dhakatimes24.com/2018/08/19/93465/ডায়রিয়ায়-আর্কান্ত-হয়ে-পুলিশ-কর্মকর্তার-মৃত্যু/ (accessed August 27, 2021).

23. Three people died in the same family due to diarrhoea, exorcism with tin rice out of fear. *Jamuna tv*. (2018).

24. *There Are Twice as Many Diarrhea Patients as Beds in Pirojpur Hospital*. prothom alo. (2018). Available online at: https://www.prothomalo.com/bangladesh/district/শয্যার-চেয়ে-দিগুণ-ডায়রিয়া-রোগী-পিরোজপুরের-হাসপাতালে-ভর্তি (accessed August 27, 2021).

25. One thousand people were affected by diarrhea in Pirojpur for one week. *Pirojpurtimes*. (2018) 24. Available online at: https://pirojpurtimes24.com/2018/05/29/news/86762620 (accessed August 27, 2021).

26. Two people died of diarrhea in Keraniganj, and four hundreds were affected. *Medivoicebd*. (2018). Available online at: https://medivoicebd.com/article/11327 (accessed August 27, 2021).

27. In Gaibandha, diarrhea affected more than two hundred. *Jugantor*. (2018). Available online at: https://www.jugantor.com/todays-paper/bangla-face/33736/গাইবান্ধায়-ডায়রিয়ায়-আর্কান্ত-দুশতাধিক (accessed August 27, 2021).

28. Increasing number of patients with diarrhea, treatment on the floor. (2019). Available online at: https://www.bd-pratidin.com/country/2019/05/13/423572 (accessed August 27, 2021).

29. Outbreak of diarrhea in Gazipur. deshrupantor. (2019). Available online at: https://www.deshrupantor.com/city/2019/08/01/158891 (accessed August 27, 2021).

30. In Shariatpur, 200 people in the hospital with diarrhea, the patients are getting nothing but saline. primenewsbd. (2019).

31. The number of children with diarrhea and pneumonia is increasing in the winter. *Banglanews*. (2020) 24. Available online at: https://www.banglanews24.com/health/news/bd/765592.details (accessed August 27, 2021).

32. Outbreak of diarrhea in Joypurhat, patient crowd in hospital. (2020). Available online at: https://www.banglanews24.com/health/news/bd/771797.details (accessed August 27, 2021).

33. One hundred and nineteen people died due to waterborne diseases in 21 districts. *Banglanews*. (2020) 24. Available online at: https://bangla.bdnews24.com/opinion/comment/62994 (accessed August 27, 2021).

34. Eighty eight affected by diarrhea due to flood in Kurigram, four schools under the river. (2020). Available online at: https://www.jugantor.com/covid-19/321654/বন্যায়-কুড়িগ্রামে-৫-জনের-মৃত্যু (accessed August 27, 2021).

35. Taniuchi M, Islam K, Sayeed MA, Platts-Mills JA, Islam MT, Khabir MIU, et al. Etiology of diarrhea requiring hospitalization in Bangladesh by quantitative polymerase chain reaction, 2014–2018. *Clin Infect Dis.* (2021) 73:e2493–e9. doi: 10.1093/cid/ciaa840

36. Carillo L, Gilman R, Mantle R, Nunez N, Watanabe J, Moron J, et al. Rapid detection of Vibrio cholerae O1 in stools of Peruvian cholera patients by using monoclonal immunodiagnostic kits. Loyaza cholera working group in Peru. *J Clin Microbiol.* (1994) 32:856–7. doi: 10.1128/jcm.32.3.856-857.1994

37. Albert MJ, Ansaruzzaman M, Bardhan PK, Faruque A, Faruque SM, Islam M, et al. Large epidemic of cholera-like disease in Bangladesh caused by Vibrio cholerae O139 synonym Bengal. *Lancet.* (1993) 342:387–90. doi: 10.1016/0140-6736(93)92811-7

38. Lessler J, Moore SM, Luquero FJ, McKay HS, Grais R, Henkens M, et al. Mapping the burden of cholera in sub-Saharan Africa and implications for control: an analysis of data across geographical scales. *Lancet.* (2018) 391:1908–15. doi: 10.1016/S0140-6736(17)33050-7

39. Colwell RR, Huq A, Islam MS, Aziz K, Yunus M, Khan NH, et al. Reduction of cholera in Bangladeshi villages by simple filtration. *Proc Nat Acad Sci.* (2003) 100:1051–5. doi: 10.1073/pnas.0237386100

40. Sack D. Underestimating the cholera problem and the potential for vaccination—A case for accelerating the use of cholera vaccines. *Bulletin de l'Institut Pasteur.* (1995) 93:229–35. doi: 10.1016/0020-2452(96)85756-3

41. Hasan ST, Das S, Faruque A, Khan AI, Clemens JD, Ahmed T. Taking care of a diarrhea epidemic in an urban hospital in Bangladesh: appraisal of putative causes, presentation, management, and deaths averted. *PLoS Negl Trop Dis.* (2021) 15:e0009953. doi: 10.1371/journal.pntd.0009953

frontiers | Frontiers in Public Health

TYPE Original Research
PUBLISHED 01 February 2023
DOI 10.3389/fpubh.2023.1127925

OPEN ACCESS

EDITED BY
Md Robiul Karim,
Bangabandhu Sheikh Mujibur Rahman Agricultural University, Bangladesh

REVIEWED BY
Majid Pirestani,
Tarbiat Modares University, Iran
Faham Khamesipour,
Shahid Beheshti University of Medical Sciences, Iran

*CORRESPONDENCE
Xiaojuan Qi
✉ xjqi@cdc.zj.cn
Jiang Chen
✉ jchen@cdc.zj.cn

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Infectious Diseases: Epidemiology and Prevention, a section of the journal Frontiers in Public Health

RECEIVED 20 December 2022
ACCEPTED 17 January 2023
PUBLISHED 01 February 2023

CITATION
He Y, Wang J, Zhang R, Chen L, Zhang H, Qi X and Chen J (2023) Epidemiology of foodborne diseases caused by *Salmonella* in Zhejiang Province, China, between 2010 and 2021. *Front. Public Health* 11:1127925. doi: 10.3389/fpubh.2023.1127925

Epidemiology of foodborne diseases caused by *Salmonella* in Zhejiang Province, China, between 2010 and 2021

Yue He†, Jikai Wang†, Ronghua Zhang, Lili Chen, Hexiang Zhang, Xiaojuan Qi* and Jiang Chen*

Department of Nutrition and Food Safety, Zhejiang Provincial Center for Disease Control and Prevention, Hangzhou, China

Objective: *Salmonella* infection is a common cause of bacterial foodborne diseases (FBDs) globally. In this study, we aimed to explore the epidemiological and etiological characteristics of *Salmonella* infection from 2012–2021 in Zhejiang Province, China.

Methods: Descriptive statistical methods were used to analyze the data reported by the Centers for Disease Control and Prevention at all levels in Zhejiang Province through the China National Foodborne Diseases Surveillance Network from 2012–2021.

Results: A total of 11,269 *Salmonella* cases were reported, with an average positive rate of 3.65%, including 1,614 hospitalizations. A significant seasonal trend was observed for *Salmonella* cases, with the highest rate over the summer period, peaking from May to October, accounting for 77.96%. The results indicated a higher positive rate among respondents aged 0–4 years, especially for the scattered children ($P < 0.05$). The highest number of *Salmonella* infections were caused due to contaminated fruit and fruit products. Households (54.69%) had the most common exposure settings. Serotypes analysis revealed that *Salmonella typhimurium* (36.07%), *Salmonella enteritidis* (15.17%), and *Salmonella london* (6.05%) were the dominant strains among the 173 serotypes. Diarrhea, abdominal pain, fever, nausea, and vomiting were the main symptoms of these serotypes.

Conclusions: FBDs caused by *Salmonella* are important issues for public health in Zhejiang Province, and there is a need to focus on the epidemiological and etiological characteristics to control *Salmonella* infections.

KEYWORDS

epidemiology, public health, foodborne disease (FBD), surveillance system, *Salmonella*

1. Introduction

Foodborne diseases (FBDs) represent global public health issues that result in considerable morbidity and mortality in all age groups and are a hurdle to socioeconomic development. The World Health Organization (WHO) estimated that there were ~600 million (almost 1 in 10) cases caused by contaminated food, resulting in 33 million disability-adjusted life years (DALYs) in 2010 (1, 2). Generally, FBDs occur due to specific pathogens, such as the bacteria, viruses, parasites, fungi, and mycotoxins, and prions, environmental factors like contamination during the production, processing, transport, and storage phases, as well as the conditions of the host's immune system (3, 4). The most frequent causes of FBDs worldwide are bacterial pathogens, the most important being *Salmonella*, *Vibrio parahaemolyticus*, *Listeria monocytogenes*, *Staphylococcus aureus*, and some other pathogens (4–8).

Salmonella, a vital microorganism responsible for FBDs, mainly exists in foods of animal origin, especially raw poultry, raw meat, eggs, and their products (9, 10). Annually, *Salmonella* causes ∼200 million to over 1 billion infections worldwide, with 93 million cases of gastroenteritis and 155,000 deaths, and 85% of illnesses which are food-linked (11–13). Due to cross-contamination in the production process, the bacteria may get transferred onto these products (14). Meat and poultry products were a good environment for the growth of *Salmonella* because of rich content of nutrients and water (15). Besides, fruit and vegetable products contaminated by animal fecal flora could act as a breeding ground for *Salmonella* (16). Moreoover, in 2017, 10,000 cases of *Salmonella* infections were registered in Poland, and the incidence rate per 100 thousand population was 26.0% (17). Human challenge studies have demonstrated that patients can develop food poisoning after ingesting *Salmonella*, which has influenza-like symptoms including nausea, vomiting, abdominal pain, and diarrhea (18). At present, *Salmonella* has more than 2,500 serotypes worldwide, and more than 200 serotypes have been identified in China. During 1996–2014, *S. typhimurium*, *S. enteritidis*, and *S. newport* were the three most common serotypes reported by the Foodborne Diseases Active Surveillance Network (FoodNet) sites of the Centers for Diseases Control and Prevention (CDC) in the US (19).

FBDs surveillance aims to monitor food contamination and harmful factors, and reduce the burden of illness due to contaminated food. There are several different types of FBD surveillance systems, including event-based surveillance, indicator-based surveillance, and integrated food chain surveillance (20). Since 2011, China has successively established a web based FBD surveillance platform, which includes the Foodborne Disease Outbreaks Surveillance System (FDOSS), Foodborne Disease Surveillance and Reporting System (FDSRS), National Molecular Traceability Network for Foodborne Diseases (TraNet), and other surveillance systems (21). The FDSRS system is applied for collecting information about foodborne disease patients visiting medical institutions at all levels, including self-reported suspicious food exposure and pathogen detection results. Zhejiang Province, on the southeast coast of China, is located at 27°02'N to 31°11'N and 118°01'E to 123°10'E, has 35,100 health institutions (including village clinics), with 1,486 hospitals and 103 CDCs (22). In this study, the national FBDs surveillance data collected over 10 years were used to describe the epidemiological characteristics, food vehicles, and setting distribution of foodborne gastroenteritis caused due to *Salmonella* infections in Zhejiang Province.

2. Methods

2.1. Diagnostic criteria for *Salmonella* FBDs

The diagnostic criteria were mainly based on the clinical symptoms and microbiological evidence. Suspected cases were considered to have an acute gastrointestinal illness (AGI) if they met one or both of the following clinical symptoms: (1) diarrhea, defined as three or more loose stools within 24 h, accompanied by abnormal fecal characteristics, and (2) vomiting (accompanied by content). Microbiological evidence was obtained when *Salmonella* was isolated from suspected food items, equipment, utensils, or when a simultaneous serotype of *Salmonella* was detected in the vomit or feces of multiple patients.

2.2. Data collection

The Zhejiang Provincial CDC (ZJCDC) has been collecting FBD- relevant data through the China National Foodborne Diseases Surveillance Network (NFDSN) since 2012. One hundred and one hospitals were asked to detect *Salmonella* pathogens and their corresponding subtypes for all suspected foodborne disease cases, and reported illnesses through NFDSN since 2012. In this study, cases reported by 101 hospitals in Zhejiang Province between 2012 and 2021 were included. Epidemiologists from the health departments first conducted an investigation to ascertain the full extent of the foodborne illness, and the information collected for each case includes the reporting region, date of occurrence, setting, etiology, food categories, number of illnesses/hospitalizations, and other details. Unknown etiology refers to foodborne disease cases in which the confirmed etiology has not been identified. Settings were classified into eight categories. Food items were identified as sources of disease through epidemiological or laboratory methods and were classified into 14 categories. Food that could not be determined was classified as "Unknown." The GIS map data of Zhejiang Province was downloaded from the national basic geographic information center of China (http://bzdt.ch.mnr.gov.cn/).

2.3. Standard laboratory protocol for *Salmonella*

Fresh stool specimens or anal swabs were collected from cases. The best specimens were a fecal specimen, anal swab was used only when the patient had no stool specimen. Specimens collected were tested as soon as possible. Specimens placed in the culture-Blair medium were tested within 24 h of refrigeration. Fresh fecal samples were placed in clean, dry containers without soap or disinfectant residue, and sent for examination within 8 h of refrigeration.

Isolation and identification of *Salmonella* were performed as described in the Operation Procedure for *Salmonella* Inspection in the Foodborne Disease Surveillance Work Manual of the National Center for Food Safety Risk Assessment. In brief, the above specimens were placed in SBG augmenting solution and cultured at 36°C for 18 to 24 h. Furthermore, after gently shaking the expanding liquid tube we applied 1 ring line to the *Salmonella* chromogenic medium or XLD AGAR plate and incubated it at 36 ± 1°C for 18 to 24 h. We picked three to five suspected colonies, inoculated in TSI AGAR, lysine decarboxylase, and nutrient AGAR plates, at 36 ± 1°C for 18 to 24 h. A single colony was scraped from a nutrient AGAR plate for systematic biochemical identification. Either of biochemical identification kit or automatic microbial biochemical identification system can be selected for identification.

The *Salmonella* serovar was identified with specific O and H antiserum samples according to the Kauffmann–White scheme as described in the instructions provided by the manufacturer of the antiserum samples (Statens Serum Institute, SSI).

2.4. Data analysis

All the data was analyzed using IBM SPSS Statistics for Windows, version 22.0 (IBM Crop., Armonk, NY, USA). Open-source software QGIS (Quantum GIS version 3.22.9) was used to map the spatial distribution of cases with positive detection rates caused by *Salmonella* between 2012 and 2021.

The total positive detection and hospitalization rates were calculated for *Salmonella*, and a linear trend test was used to detect the change in the positive detection and hospitalization rates annually. Chi-square tests were used to compare the relationship between demographic characteristics and the positive rate, including sex, age, annual distribution, season, and area. Fisher's exact test was used if the conditions were not met in the chi-square test. A *post-hoc* test was used for pairwise comparisons. The seasons were classified as winter (December to February), spring (March to May), summer (June to August), and autumn (September to November). $P < 0.05$ was considered as significant.

3. Results

3.1. General epidemiological characteristics

Between 2012 and 2021, 420,736 suspected FBD cases were reported in medical institutions at all levels in 11 cities in Zhejiang Province, and 308,326 stool samples were collected for *Salmonella* testing. The total positive rate was 3.65% (11,269/3,08,326). The positive detection rate for *Salmonella* increased from 1.69 to 6.61% during 2012–2021 (Table 1, Figure 1), and the number of reported confirmed cases increased, especially in 2020 (6.80%) and 2021 (6.61%) (Table 1). A significant increase in the hospitalization rate was observed during the study period (Table 1, Figure 1).

The regional distribution of cases with positive *Salmonella* infection among 11 cities is shown in Figure 2. City of Taizhou, Quzhou, and Lishui cities had a positive rate of 6.57% (1,907 cases), 4.54% (820 cases), and 4.39% (1,173 cases), respectively (Table 1, Figure 2). Whereas, city of Jinhua, Quzhou, and Lishui cities had the highest hospitalization rates with 17.22, 16.17, and 14.13%, respectively.

3.2. Trend and seasonality

In terms of temporal distribution, *Salmonella* infection mainly occurred seasonally from May to October, during which 8,754 cases occurred, accounting for 77.69% of the total cases. These months are the hottest in Zhejiang, with average temperature ranging between 20.7 and 28.2°C (Figure 3) (23). Moreover, the highest positive rate (5.21%) was observed in summer (June to August) ($P < 0.001$) (Table 1).

3.3. Age, gender, and occupational difference

The average age of 11,269 patients (6,155 males and 5,114 females) was 33.63 years. A slight difference was observed between the different sex groups ($P = 0.012$), as shown in Table 1. As for age distribution, the majority of reported *Salmonella* cases affected children aged 0–4 years (4,060 cases, 36.02%), and older adults aged >60 years (2,109 cases, 18.72%), with positive rates of 8.79 and 4.34%, and hospitalization rates of 48.14 and 27.70%, respectively. A significant occupational difference was observed between the occupational groups ($P < 0.001$). The positive infection rate was the highest in scattered children (3,136 cases, 13.02%), with a hospitalization rate of 37.73%.

3.4. Implicated foods and settings

Among the 11,269 *Salmonella* cases, 1,434 (12.73%) were attributed to fruits and fruit products (Table 2). Aquatic products (1,370 cases, 12.16%), meat and meat products (1,337 cases, 11.86%), cereals and grain products (1,054 cases, 9.35%), milk and dairy products (705 cases, 6.26%), vegetables and vegetable products (657 cases, 5.83%), eggs and egg products (493 cases, 4.37%), beverages and frozen drinks (273 cases, 2.42%), infant foods (226 cases, 2.01%), and beans and soy products (186 cases, 1.65%), these were the most commonly reported food items. Approximately 6.85% (772/11,269) of the cases were associated with mixed dishes, 6.05% (682/11,269) with multiple foods, and 12.66% (1,427/11,269) with unknown food. In addition, 653 (5.79%) cases were relevant to other food products containing liquor products, fungi, nuts, sweets, and water. Among single food category, fruit and fruit products (186 hospitalizations, 11.52%) were responsible for most hospitalizations, followed by meat and meat products (168 hospitalizations, 10.41%) and cereals and grain products (156 hospitalizations, 9.67%).

The distribution of cases according to the setting is shown in Table 2. *Salmonella* FBDs occurred most frequently in household settings (6,163 cases, 54.69%), followed by restaurants (479 cases, 4.25%), retail (182 cases, 1.61%), collective canteen (142 cases, 1.26%), schools (30 cases, 0.27%), rural banquets (28, 0.25%), and other settings (4,245 cases, 37.67%), including unknown settings (1,312 cases, chophouses, street stalls, and delivering meals). *Salmonella* FBDs in households (772 hospitalizations, 47.83%), retail (36 hospitalizations, 2.23%), and restaurants (33 hospitalizations, 2.04%) resulted in a relatively high numbers of hospitalizations.

3.5. Serotypes and symptoms

In this study, 173 *Salmonella* serotypes were identified. *Salmonella typhimurium* was the most common serotype, accounting for 36.07% (4,065/11,269), and *Salmonella enteritidis* was the second, accounting for 15.17% (1,710/11,269), followed by *Salmonella london*, accounting for 6.05% (682/11,269). Among the 11,269 *Salmonella* cases, 99.41% had diarrhea, 47.22% had abdominal pain, 27.03% had fever, 20.05% had nausea, and 18.46% had vomiting. Symptoms varied greatly according to serotype. Diarrhea was the most common symptom among the serotypes (Table 3).

4. Discussion

Salmonella infection is a vital public health concern in the Zhejiang Province. In this study, we for the first time described the epidemiological and etiological characteristics of the *Salmonella*

TABLE 1 Demographic characteristics and *Salmonella* positive rate in Zhejiang Province from 2012 to 2021.

Variable	Cases		Hospitalizations[a]		Positive rate (%)	χ^2	*P*
	n	%	*n*	%			
Annual distribution						0.103	<0.001
2012	69	0.61	0	0.00	1.69		
2013	324	2.88	51	3.16	2.30		
2014	502	4.45	100	6.20	1.46		
2015	615	5.46	112	6.94	1.44		
2016	1,109	9.84	159	9.85	2.54		
2017	1,078	9.57	193	11.96	3.36		
2018	1,296	11.50	199	12.33	4.01		
2019	1,502	13.33	162	10.04	4.45		
2020	2,234	19.82	289	17.91	6.80		
2021	2,540	22.54	349	21.62	6.61		
Area						0.065	<0.001
Hangzhou	1,306	11.59	40	2.48	2.78		
Ningbo	1,181	10.48	150	9.29	3.74		
Wenzhou	1,050	9.32	108	6.69	2.48		
Jiaxing	788	6.99	77	4.77	3.35		
Huzhou	357	3.17	94	5.82	1.68		
Shaoxing	899	7.98	136	8.43	4.29		
Jinhua	1,252	11.11	278	17.22	3.80		
Quzhou	820	7.28	261	16.17	4.54		
Zhoushan	536	4.76	85	5.27	3.57		
Taizhou	1,907	16.92	157	9.73	6.57		
Lishui	1,173	10.41	228	14.13	4.39		
Season						0.079	<0.001
Spring	2,052	18.21	310	19.21	3.32		
Summer	5,660	50.23	799	49.50	5.21		
Autumn	3,094	27.46	432	26.77	3.66		
Winter	463	4.11	73	4.52	0.87		
Sex						6.275	0.012
Male	6,155	54.62	928	57.50	3.74		
Female	5,114	45.38	686	42.50	3.57		
Age (year)						0.005	<0.001
0–4	4,060	36.02	777	48.14	8.79		
5–14	607	5.39	109	6.75	3.25		
15–24	729	6.47	42	2.60	1.80		
25–44	1,999	17.74	84	5.20	2.03		
45–59	1,765	15.66	155	9.60	3.22		
≥60	2,109	18.72	447	27.70	4.34		
Occupation						0.007	<0.001
Farmer	3,008	26.69	435	26.95	3.44		
Scattered kids	3,136	27.83	609	37.73	13.02		

(Continued)

TABLE 1 (Continued)

Variable	Cases		Hospitalizations[a]		Positive rate (%)	χ^2	*P*
	n	%	*n*	%			
Worker	684	6.07	40	2.48	3.08		
Student	701	6.22	77	4.77	2.39		
Official staff	408	3.62	18	1.12	1.62		
Unemployed	560	4.97	66	4.09	2.53		
Kids in kindergarten	1,106	9.81	223	13.82	4.47		
Retirees	367	3.26	73	4.52	3.01		
Others	1,089	9.66	58	3.59	1.98		
Unknown	210	1.86	15	0.93	2.09		

[a]Hospitalization of cases with positive detection results.

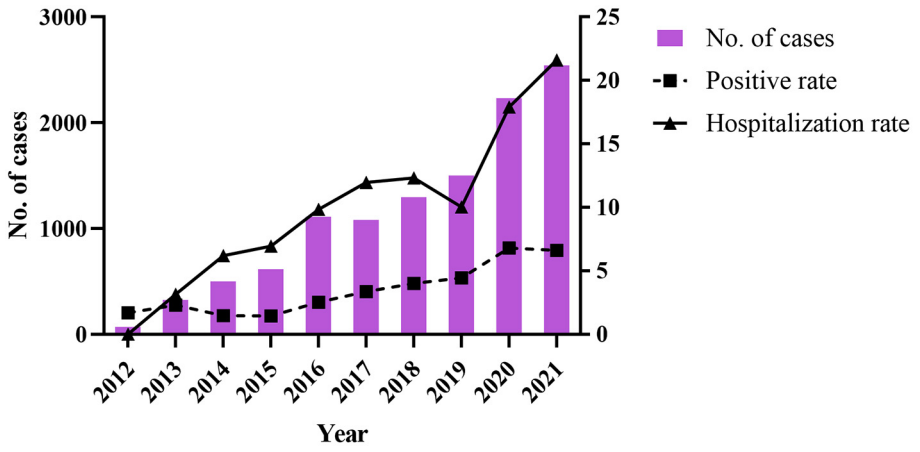

FIGURE 1
The change of number of cases, positive rate, and hospitalization rate of *Salmonella* during 2012–2021.

infection in the Zhejiang Province between 2012 and 2021. During the years, 11,269 cases with 1,614 (14.32%) hospitalizations were reported, corresponding to an average positive rate of 3.65% for the whole province. The average age of patients infected with *Salmonella* was 33.63 years. In all settings and food categories, *Salmonella* cases occurred most commonly in household settings (6,163 cases, 54.69%) due to fruit and fruit products (1,434 cases, 12.73%).

The positive rate of *Salmonella* infection increased during 2012–2021 and remained particularly high between 2020 and 2021. Considering the gradual improvement of the surveillance system at all levels of CDCs and hospitals, more attention has been paid to FBDs and cases have been reported in detail (24). Compared to the rates abroad, the CDC estimated that *Salmonella enterica* caused 1.2 million infections, 24,000 hospitalizations, and 450 deaths in the United States (25). According to the European Food Safety Authority (EFSA) and European CDC (ECDC) reports, 88,715 confirmed cases of *Salmonella* infection and an EU notification rate of 23.4 cases per 100,000 population were recorded (26). South East Asia, with 11 different countries, ranks third as the super region for the global burden of *Salmonella*-induced gastroenteritis (27). Some epidemiological studies have revealed the prevalence, characterization, genetic investigation, serovar distribution, and antibiotic resistance in China, however, the results remain ambiguous (28–30). Therefore, it is reasonable to assume that FBD caused due to *Salmonella* infection is a growing public health issue in the Zhejiang Province.

Salmonella infection showed an obvious pattern according to age, and young children and older adults were especially vulnerable. Following are some plausible explanations as to why young children and older adults are more susceptible to *Salmonella* infection. Primarily, immunocompromised children and older adults and those with underlying conditions are particularly vulnerable to invasive diseases (31, 32). Due to their immature immune systems and permeable gastrointestinal tracts, infants and young children are more susceptible to infection by foodborne pathogenic bacteria than other age groups (33). Older adults exhibit dysregulated immune responses to pathogens. In addition, consumption of infant formula contaminated with *Salmonella* may result in serious illness. In terms of community risks, powdered infant formula contamination and its associated hazards may not be fully recognized (34, 35). Moreover, parents pay high attention to *Salmonella* infection, and they tend to seek medical advice (36).

Regional differences in the distribution of *Salmonella* were observed in the present study. Considering the location of the Zhejiang Province, the annual mean temperature ranges from 15.0 to 18.0°C and the province experience a subtropical humid climate.

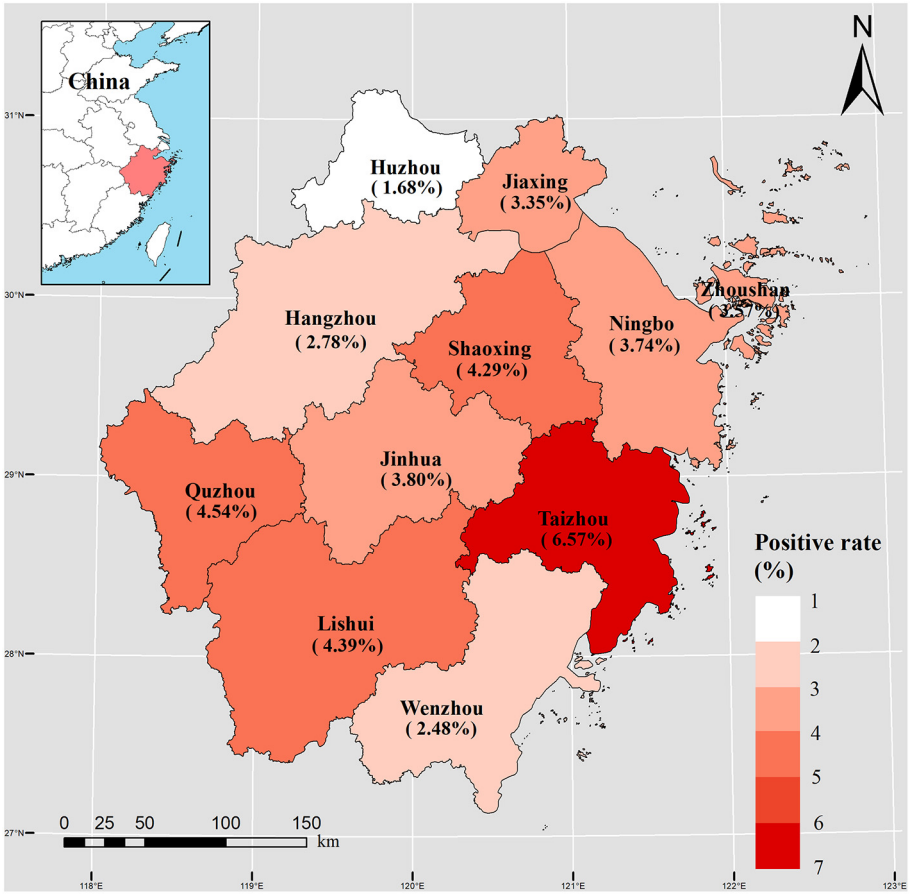

FIGURE 2
Spatial distribution of 11,269 *Salmonella* cases during 2012–2021 in 11 cities of Zhejiang Province.

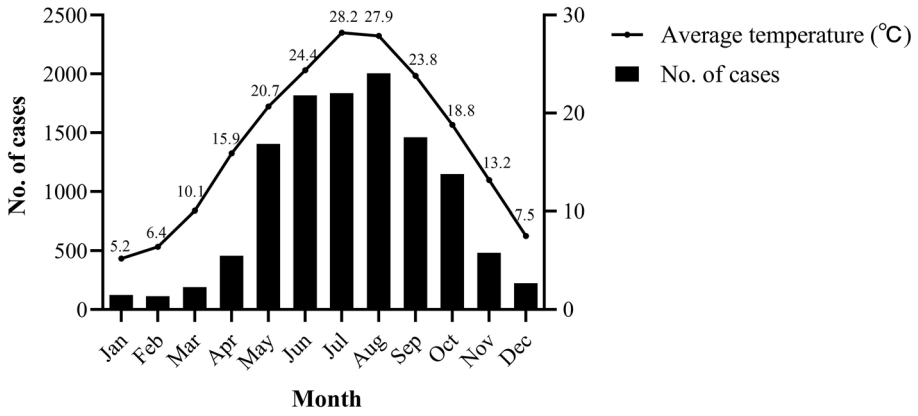

FIGURE 3
Temporal distribution and number of *Salmonella* cases, by month of occurrence, 2012–2021.

Taizhou was deemed to have the highest rate of *Salmonella* positivity, with a population of more than six million and 73.0% mountainous area. Hangzhou, with the most cases of *Salmonella* infection, has the lowest percentage of hospitalizations. To the best of our knowledge, in many locations with limited resources, food safety methods for prevention are rarely the main focus, and the lack of food safety knowledge is a vital reason for FBDs (37). With the diverse species of *Salmonella* serotypes, there are differences in the biofilm lifestyles, long-term persistence outside, and immune responses (38). Recent studies have revealed that climate and seasonality may play important roles in the prevalence of *Salmonella* (39). In this study, *Salmonella* infection showed a significantly increased positive rates in the warm seasons, especially in summer. Stronger research evidence indicated that *Salmonella* infections are elevated in warm climates (40, 41). Owing to high temperatures, people prefer raw and cold foods. Frozen, raw, and cold foods, such as meat, milk and milk products,

TABLE 2 Food and Setting distribution of *Salmonella* positive cases in Zhejiang Province from 2012 to 2021.

Variables	Case		Hospitalizations	
	n	%	*n*	%
Food				
Fruits and fruit products	1,434	12.73	186	11.52
Aquatic products	1,370	12.16	126	7.81
Meat and meat products	1,337	11.86	168	10.41
Cereals and grain products	1,054	9.35	156	9.67
Milk and dairy products	705	6.26	118	7.31
Vegetables and vegetable products	657	5.83	86	5.33
Eggs and egg products	493	4.37	80	4.96
Beverages and frozen drinks	273	2.42	18	1.12
Infant foods	226	2.01	36	2.23
Beans and soy products	186	1.65	26	1.61
Mixed dishes	772	6.85	97	6.01
Multiple foods	682	6.05	124	7.68
Unknown	1,427	12.66	282	17.47
Others	653	5.79	111	6.88
Setting				
Household	6,163	54.69	772	47.83
Restaurant	479	4.25	33	2.04
Retail	182	1.62	36	2.23
Collective canteens	142	1.26	7	0.43
School	30	0.27	8	0.50
Rural banquet	28	0.25	2	0.12
Unknown	1,525	13.53	309	19.14
Others	2,720	24.14	490	30.36

have been identified as risk factors for *Salmonella* infection (42). Furthermore, warm and suitable temperatures are more suitable for the growth of *Salmonella*. Therefore, refrigerating foods is necessary for the prevention and control of bacterial FBDs.

Interestingly, more than half of the *Salmonella* positive cases occurred in household setting (6,163 case, 54.69%) in our study. According to a survey of six European countries, approximately 40% of foodborne infections are acquired at home because of cross-contamination and food preferences (43). The food category results demonstrated that fruits and fruit products (1,434 cases, 12.73%), aquatic products (1,370 cases, 12.16%), and meat and meat products (1,337 cases, 11.86%) acquired the top three positions among all food categories in Zhejiang Province that caused *Salmonella* infections. The main sources of *Salmonella* infection in humans are meat products, including the consumption of contaminated poultry meat at the global level (44). A systematic review and meta-analysis had evaluated that the prevalence level differed from high to low among raw poultry meat, including chicken, pigeon, duck, and other poultry meat (9). However, an increasing number of reports have linked *Salmonella* contaminated raw vegetables and fruits with food poisoning (45). *Salmonella* uses multiple strategies to manipulate the host defense system while in contact with fruits and vegetables, including affecting the genetic variation, controlling the heterogenous expression of flagellin, and suppressing the dual expression of effector proteins (46).

Serotyping results demonstrated that most *Salmonella* FBDs were caused by multiple serotypes, including *S. typhimurium*, *S. enteritidis*, and *S. london*, which is consistent with previous studies (47, 48). In the past two decades, *S. typhimurium* and *S. enteritidis* have become the most common *Salmonella* serotypes responsible for human infections in different regions (49). An epidemiological investigation showed that high levels of *Salmonella* contamination were detected in meat products, and multiple virulence-associated genes were isolated in Southern China, Guangdong Province (50). Some valuable baseline data collected from other provincial regions also showed significant differences in *Salmonella* serotypes (51, 52). It is noteworthy that *S. derby*, *S. risoson*, *S. stanley*, *S. dublin*, *S. gold coast*, *S. paratyphi*, *S. rosenticus*, *S. infantis*, and *S. sick cattle* were also considerable serovars, accounting for 1.86, 1.32, 1.30, 1.29, 1.22, 1.19, 1.14, 1.10, and 1.04% of the total serotypes, respectively. Moreover, *S. stanley*, *S. dublin*, and *S. sick bovine* were higher in the city of Jinhua, Quzhou, and Zhoushan, respectively, which indicated that there were some differences in the distribution of the dominant serotypes. These differences may be associated with geographical location, eating habits, climatic conditions, and food preferences. Therefore, it is essential to systematically monitor the *Salmonella* serotype distribution through a proper sampling layout.

In terms of clinical symptoms, our results showed that most serotypes could cause AGI symptoms, including diarrhea, abdominal pain, fever, nausea, and vomiting. The twelve main serotypes caused diarrhea in more than 90% of cases. The proportion of abdominal pain was the highest in *S. dublin* (60.69%) and lowest in *S. stanley* (39.04%). Remarkably, *S. dublin* (46.21%) was responsible for the highest fever proportion, whereas it was lowest in *S. rosenticus* (13.28%). Nevertheless, fever caused by *Salmonella* infection may be difficult to distinguish from other febrile diseases; therefore, etiological examination is essential (18). The highest proportions of nausea and vomiting were caused by S. *dublin*. *S. dublin* mainly colonizes cattle; however, upon infection, it might lead to invasive illness in humans (53).

This study had some limitations. First of all, the case data were collected through the NFDSN, which is a passive surveillance system and some information was either missing or incomplete, such as food categories, settings and etc., so the conclusions might not be representative of unknown classification. Second, although our surveillance system has improved significantly since 2012 in all province, the data quality is still related to regional distribution, local economic level, detection capacity, and coordination degree. Additionally, food information was self-reported by patients, so there was great uncertainty regarding epidemiological tracing. Further case surveillance should focus on the etiology and food, and also training investigators to make efforts to obtain the exact causes of FBDs and accurate characteristics.

TABLE 3 Reported signs and symptoms of *Salmonella* cases in different serotypes.

Variables	Typhimurium (*N* = 4,065)	Enteritidis (*N* = 1,710)	London (*N* = 682)	Derby (*N* = 210)	Risson (*N* = 149)	Stanley (*N* = 146)	Dublin (*N* = 145)	Gold Coast (*N* = 137)	Paratyphi (*N* = 134)	Rosenticus (*N* = 128)	Infantis (*N* = 124)	Sick cattle (*N* = 117)
Diarrhea	4,012 (98.70)	1,688 (98.71)	674 (98.83)	210 (100.00)	146 (97.99)	145 (99.32)	145 (100.00)	136 (99.27)	133 (99.25)	126 (98.44)	123 (99.19)	111 (94.87)
Abdominal pain	1,784 (43.89)	944 (55.20)	381 (55.87)	82 (39.05)	76 (51.01)	57 (39.04)	88 (60.69)	81 (59.12)	64 (47.76)	54 (42.19)	56 (45.16)	57 (48.72)
Fever (≥37.5°C)	1,204 (29.62)	458 (26.78)	136 (19.94)	38 (18.10)	36 (24.16)	37 (25.34)	67 (46.21)	31 (22.63)	21 (15.67)	17 (13.28)	34 (27.42)	21 (17.95)
Vomiting	688 (16.92)	408 (23.86)	116 (17.01)	42 (20.00)	22 (14.77)	28 (19.18)	48 (33.10)	19 (13.87)	22 (16.42)	24 (18.75)	19 (15.32)	17 (14.53)
Nausea	675 (16.61)	427 (24.97)	156 (22.87)	46 (21.90)	33 (22.15)	27 (18.49)	42 (28.97)	27 (19.71)	21 (15.67)	26 (20.31)	25 (20.16)	16 (13.68)
Debilitation	269 (6.62)	157 (9.18)	55 (8.06)	16 (7.62)	14 (9.40)	12 (8.22)	11 (7.59)	11 (8.03)	13 (9.70)	7 (5.47)	13 (10.48)	7 (5.98)
Thirsty	115 (2.83)	77 (4.50)	20 (2.93)	6 (2.86)	5 (3.36)	7 (4.79)	3 (2.07)	4 (2.92)	8 (5.97)	3 (2.34)	3 (2.42)	5 (4.27)
Hypourocrinia	113 (2.78)	51 (2.98)	22 (3.23)	8 (3.81)	2 (1.34)	4 (2.74)	6 (4.14)	4 (2.92)	7 (5.22)	1 (0.78)	3 (2.42)	4 (3.42)
Dehydration	94 (2.31)	26 (1.52)	11 (1.61)	3 (1.43)	2 (1.34)	5 (3.42)	2 (1.38)	2 (1.46)	2 (1.49)	1 (0.78)	3 (2.42)	1 (0.85)
Tenesmus	51 (1.25)	17 (0.99)	6 (0.88)	2 (0.95)	1 (0.67)	3 (2.05)	3 (2.07)	3 (2.19)	1 (0.75)	2 (1.56)	4 (3.23)	0 (0.00)
Shiver	28 (0.69)	15 (0.88)	5 (0.73)	0 (0.00)	6 (4.03)	3 (2.05)	2 (1.38)	0 (0.00)	0 (0.00)	1 (0.78)	1 (0.81)	2 (1.71)
Flushed face	26 (0.64)	16 (0.94)	3 (0.44)	1 (0.48)	1 (0.67)	2 (1.37)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.85)
Pale	25 (0.62)	8 (0.47)	2 (0.29)	3 (1.43)	1 (0.67)	2 (1.37)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Headache	18 (0.44)	17 (0.99)	4 (0.59)	1 (0.48)	0 (0.00)	0 (0.00)	2 (1.38)	0 (0.00)	0 (0.00)	1 (0.78)	2 (1.61)	0 (0.00)
Weight loss	15 (0.37)	6 (0.35)	2 (0.29)	1 (0.48)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)

10.3389/fpubh.2023.1127925

5. Conclusion

We have demonstrated for the first time of epidemiological characteristics for foodborne diseases caused by *Salmonella* in China over the past 10 years. Since *Salmonella* infections continues to be a severe public health concern worldwide, we recommend that the data accuracy of food collection for suspected exposure should be optimized and compared with the actual contamination results in food items to provide support for supervision. To prevent and control future FBDs caused by *Salmonella*, it is necessary to carry out drug resistance analysis and whole genome sequencing of *Salmonella* cases, and further explore its biological mechanism. There is a need to carry out an overall assessment of *Salmonella* infection in residents by strengthening FBDs surveillance, source attribution and burden estimation, and more efforts should be directed toward conducting comprehensive assessments for specific public health policy formulation.

Data availability statement

The datasets presented in this article are not readily available because Ethics Committee of Zhejiang Provincial Center for Disease Control and Prevention. Requests to access the datasets should be directed to xjqi@cdc.zj.cn.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Zhejiang Provincial Center for Disease Control and Prevention. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

Conceptualization and writing—review and editing: YH and XQ. Data curation: JW. Investigation: LC. Methodology: HZ and YH. Project administration: XQ and JC. Supervision: JC. Validation: RZ. Writing—original draft: YH. All authors have read and agreed to the published version of the manuscript.

Funding

This work was supported by Medical and Health Science and Technology Project of Zhejiang Province [grant number: 2022KY712], Medical and Health Science and Technology Project of Zhejiang Province [grant number: 2022KY717], and Medical and Health Science and Technology Project of Zhejiang Province [grant number: 2021KY624].

Acknowledgments

We thank the CDCs at all level of Zhejiang Province for the efforts of more than 10 years, which provided a large amount of data to analyze the epidemic characteristics. It is of great significance to prevent the occurrence of FBDs and protect the health of people.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Faour-Klingbeil D, E CDT. Prevention and control of foodborne diseases in Middle-East North African countries: review of national control systems. *Int J Environ Res Public Health*. (2019) 17:70. doi: 10.3390/ijerph17010070

2. Torgerson PR, Devleesschauwer B, Praet N, Speybroeck N, Willingham AL, Kasuga F, et al. World health organization estimates of the global and regional disease burden of 11 foodborne parasitic diseases, 2010: a data synthesis. *PLoS Med*. (2015) 12:e1001920. doi: 10.1371/journal.pmed.1001920

3. Gallo M, Ferrara L, Calogero A, Montesano D, Naviglio D. Relationships between food and diseases: what to know to ensure food safety. *Food Res Int*. (2020) 137:109414. doi: 10.1016/j.foodres.2020.109414

4. Martinović T, Andjelković U, Gajdošik M, Rešetar D, Josić D. Foodborne pathogens and their toxins. *J Proteomics*. (2016) 147:226–35. doi: 10.1016/j.jprot.2016.04.029

5. Pucciarelli MG, García-Del Portillo F. *Salmonella* intracellular lifestyles and their impact on host-to-host transmission. *Microbiol Spectrum*. (2017) 5:2016. doi: 10.1128/microbiolspec.MTBP-0009-2016

6. Dutta D, Kaushik A, Kumar D, Bag S. Foodborne pathogenic vibrios: antimicrobial resistance. *Front Microbiol*. (2021) 12:638331. doi: 10.3389/fmicb.2021.638331

7. Jordan K, McAuliffe O. Listeria monocytogenes in foods. *Adv Food Nutr Res*. (2018) 86:181–213. doi: 10.1016/bs.afnr.2018.02.006

8. Kadariya J, Smith TC, Thapaliya D. Staphylococcus aureus and staphylococcal food-borne disease: an ongoing challenge in public health. *Biomed Res Int*. (2014) 2014:827965. doi: 10.1155/2014/827965

9. Sun T, Liu Y, Qin X, Aspridou Z, Zheng J, Wang X, et al. The prevalence and epidemiology of *Salmonella* in retail raw poultry meat in china: a systematic review and meta-analysis. *Foods*. (2021) 10:2757. doi: 10.3390/foods10112757

10. Whiley H, Ross K. *Salmonella* and eggs: from production to plate. *Int J Environ Res Public Health*. (2015) 12:2543–56. doi: 10.3390/ijerph120302543

11. Chlebicz A, Slizewska K. *Campylobacteriosis, Salmonellosis, Yersiniosis*, and *Listeriosis* as Zoonotic foodborne diseases: a review. *Int J Environ Res Public Health*. (2018) 15:863. doi: 10.3390/ijerph15050863

12. Castro-Vargas RE, Herrera-Sánchez MP, Rodríguez-Hernández R, Rondón-Barragán IS. Antibiotic resistance in *Salmonella* spp. Isolated from poultry: a global overview. *Vet world.* (2020) 13:2070–84. doi: 10.14202/vetworld.2020.2070-2084

13. Hung YT, Lay CJ, Wang CL, Koo M. Characteristics of non-typhoidal *Salmonella*g astroenteritis in Taiwanese children: a 9-year period retrospective medical record review. *J Infect Public Health.* (2017) 10:518–21. doi: 10.1016/j.jiph.2016.09.018

14. Yeh Y, Purushothaman P, Gupta N, Ragnone M, Verma SC, de Mello AS. Bacteriophage application on red meats and poultry: effects on *Salmonella* population in final ground products. *Meat Sci.* (2017) 127:30–4. doi: 10.1016/j.meatsci.2017.01.001

15. Wessels K, Rip D, Gouws P. *Salmonella* in chicken meat: consumption, outbreaks, characteristics, current control methods and the potential of bacteriophage use. *Foods*. (2021) 10:1742. doi: 10.3390/foods10081742

16. Kore K, Asrade B, Demissie K, Aragaw K. Characterization of *Salmonella* isolated from apparently healthy slaughtered cattle and retail beef in Hawassa, Southern Ethiopia. *Prev Vet Med.* (2017) 147:11–6. doi: 10.1016/j.prevetmed.2017.08.018

17. Milczarek M, Sadkowska-Todys M, Czarkowski MP, Kitowska W. *Salmonellosis* in Poland in 2017. *Przegl Epidemiol.* (2019) 73:463–77. doi: 10.32394/pe.73.44

18. Crump JA, Sjölund-Karlsson M, Gordon MA, Parry CM. Epidemiology, clinical presentation, laboratory diagnosis, antimicrobial resistance, and antimicrobial management of invasive *Salmonella* infections. *Clin Microbiol Rev.* (2015) 28:901–37. doi: 10.1128/CMR.00002-15

19. Powell MR, Crim SM, Hoekstra RM, Williams MS, Gu W. Temporal patterns in principal *Salmonella* serotypes in the USA 1996–2014. *Epidemiol Infect.* (2018) 146:437–41. doi: 10.1017/S0950268818000195

20. Ford L, Miller M, Cawthorne A, Fearnley E, Kirk M. Approaches to the surveillance of foodborne disease: a review of the evidence. *Foodborne Pathog Dis.* (2015) 12:927–36. doi: 10.1089/fpd.2015.2013

21. Chen L, Sun L, Zhang R, Liao N, Qi X, Chen J. Surveillance for foodborne disease outbreaks in Zhejiang Province, China, 2015–2020. *BMC Public Health.* (2022) 22:135. doi: 10.1186/s12889-022-12568-4

22. Zhejiang Provincial Bureau of Statistics. Available online at: http://tjj.zj.gov.cn/ (accessed December 15, 2022).

23. Sun L, Zhang H, Chen J, Chen L, Qi X, Zhang R. Epidemiology of foodborne disease outbreaks caused by non-typhoidal *Salmonella* in Zhejiang Province, China, 2010–2019. *Foodborne Pathog Dis.* (2021) 18:880–6. doi: 10.1089/fpd.2021.0006

24. Chen L, Wang J, Zhang R, Zhang H, Qi X, He Y, et al. An 11-year analysis of bacterial foodborne disease outbreaks in Zhejiang Province, China. *Foods*. (2022) 11:2382. doi: 10.3390/foods11162382

25. Harvey RR, Friedman CR, Crim SM, Judd M, Barrett KA, Tolar B, et al. Epidemiology of *Salmonella* enterica Serotype Dublin Infections among Humans, United States, 1968–2013. *Emerg Infect Dis.* (2017) 23:1493–501. doi: 10.3201/eid2309.170136

26. Bonardi S. *Salmonella* in the pork production chain and its impact on human health in the European Union. *Epidemiol Infect.* (2017) 145:1513–26. doi: 10.1017/S095026881700036X

27. Patra SD, Mohakud NK, Panda RK, Sahu BR, Suar M. Prevalence and multidrug resistance in *Salmonella* enterica Typhimurium: an overview in South East Asia. *World J Microbiol Biotechnol.* (2021) 37:185. doi: 10.1007/s11274-021-03146-8

28. Shen W, Chen H, Geng J, Wu RA, Wang X, Ding T. Prevalence, serovar distribution, and antibiotic resistance of *Salmonella* spp. isolated from pork in China: a systematic review and meta-analysis. *Int J Food Microbiol.* (2022) 361:109473. doi: 10.1016/j.ijfoodmicro.2021.109473

29. Zhao X, Hu M, Zhang Q, Zhao C, Zhang Y, Li L, et al. Characterization of integrons and antimicrobial resistance in *Salmonella* from broilers in Shandong, China. *Poult Sci.* (2020) 99:7046–54. doi: 10.1016/j.psj.2020.09.071

30. Jiu Y, Meng X, Hong X, Huang Q, Wang C, Chen Z, et al. Prevalence and characterization of *Salmonella* in three typical commercial pig abattoirs in Wuhan, China. *Foodborne Pathog Dis.* (2020) 17:620–7. doi: 10.1089/fpd.2019.2737

31. Wen SC, Best E, Nourse C. Non-typhoidal *Salmonella* infections in children: review of literature and recommendations for management. *J Paediatr Child Health.* (2017) 53:936–41. doi: 10.1111/jpc.13585

32. Allen JC, Toapanta FR, Chen W, Tennant SM. Understanding immunosenescence and its impact on vaccination of older adults. *Vaccine.* (2020) 38:8264–72. doi: 10.1016/j.vaccine.2020.11.002

33. Kent RM, Fitzgerald GF, Hill C, Stanton C, Ross RP. Novel approaches to improve the intrinsic microbiological safety of powdered infant milk formula. *Nutrients.* (2015) 7:1217–44. doi: 10.3390/nu7021217

34. Blackshaw K, Valtchev P, Koolaji N, Berry N, Schindeler A, Dehghani F, et al. The risk of infectious pathogens in breast-feeding, donated human milk and breast milk substitutes. *Public Health Nutr.* (2021) 24:1725–40. doi: 10.1017/S1368980020000555

35. Yang B, Zhao H, Cui S, Wang Y, Xia X, Xi M, et al. Prevalence and characterization of *Salmonella* enterica in dried milk-related infant foods in Shaanxi, China. *J Dairy Sci.* (2014) 97:6754–60. doi: 10.3168/jds.2014-8292

36. Behera JR, Rup AR, Dash AK, Sahu SK, Gaurav A, Gupta A. Clinical and laboratory profile of enteric fever in children from a tertiary care centre in Odisha, Eastern India. *Cureus.* (2021) 13:e12826. doi: 10.7759/cureus.12826

37. Chapman B, Gunter C. Local food systems food safety concerns. *Microbiol Spect.* (2018) 6:2017. doi: 10.1128/microbiolspec.PFS-0020-2017

38. Harrell JE, Hahn MM, D'Souza SJ, Vasicek EM, Sandala JL, Gunn JS, et al. *Salmonella* biofilm formation, chronic infection, and immunity within the intestine and hepatobiliary tract. *Front Cell Infect Microbiol.* (2020) 10:624622. doi: 10.3389/fcimb.2020.624622

39. Smith BA, Meadows S, Meyers R, Parmley EJ, Fazil A. Seasonality and zoonotic foodborne pathogens in Canada: relationships between climate and *Campylobacter*, *E. coli*, and *Salmonella* in meat products. *Epidemiol Infect.* (2019) 147:e190. doi: 10.1017/S0950268819000797

40. Lake IR. Food-borne disease and climate change in the United Kingdom. *Environ Health Glob Access Sci Sou.* (2017) 16:117. doi: 10.1186/s12940-017-0327-0

41. Naumova EN, Jagai JS, Matyas B, DeMaria A Jr, MacNeill IB, Griffiths JK. Seasonality in six enterically transmitted diseases and ambient temperature. *Epidemiol Infect.* (2007) 135:281–92. doi: 10.1017/S0950268806006698

42. Morton VK, Kearney A, Coleman S, Viswanathan M, Chau K, Orr A, et al. Outbreaks of *Salmonella* illness associated with frozen raw breaded chicken products in Canada, 2015–2019. *Epidemiol Infect.* (2019) 147:e254. doi: 10.1017/S0950268819001432

43. Møretrø T, Nguyen-The C, Didier P, Maître I, Izsó T, Kasza G, et al. Consumer practices and prevalence of *Campylobacter*, *Salmonella,* and norovirus in kitchens from six European countries. *Int J Food Microbiol.* (2021) 347:109172. doi: 10.1016/j.ijfoodmicro.2021.109172

44. Antunes P, Mourão J, Campos J, Peixe L. *Salmonellosis*: the role of poultry meat. Clinical microbiology and infection. *Eur Soc Clin Microbiol Infect Dis.* (2016) 22:110–21. doi: 10.1016/j.cmi.2015.12.004

45. Wiedemann A, Virlogeux-Payant I, Chaussé AM, Schikora A, Velge P. Interactions of *Salmonella* with animals and plants. *Front Microbiol.* (2014) 5:791. doi: 10.3389/fmicb.2014.00791

46. Zarkani AA, Schikora A. Mechanisms adopted by *Salmonella* to colonize plant hosts. *Food Microbiol.* (2021) 99:103833. doi: 10.1016/j.fm.2021.103833

47. Xu H, Zhang W, Zhang K, Zhang Y, Wang Z, Zhang W, et al. Characterization of *Salmonella* serotypes prevalent in asymptomatic people and patients. *BMC Infect Dis.* (2021) 21:632. doi: 10.1186/s12879-021-06340-z

48. Kim SH, Sung GH, Park EH, Hwang IY, Kim GR, Song SA, et al. Serotype Distribution and Antimicrobial Resistance of *Salmonella* Isolates in Korea between 2016 and 2017. *Ann Lab Med.* (2022) 42:268–73. doi: 10.3343/alm.2022.42.2.268

49. Sun H, Wan Y, Du P, Bai L. The epidemiology of monophasic *Salmonella* typhimurium. *Foodborne Pathog Dis.* (2020) 17:87–97. doi: 10.1089/fpd.2019.2676

50. Chen Z, Bai J, Wang S, Zhang X, Zhan Z, Shen H, et al. Prevalence, antimicrobial resistance, virulence genes and genetic diversity of *Salmonella* Isolated from retail duck meat in Southern China. *Microorganisms.* (2020) 8:444. doi: 10.3390/microorganisms8030444

51. Li Y, Yang X, Zhang H, Jia H, Liu X, Yu B, et al. Prevalence and antimicrobial susceptibility of *Salmonella* in the commercial eggs in China. *Int J Food Microbiol.* (2020) 325:108623. doi: 10.1016/j.ijfoodmicro.2020.108623

52. Zheng D, Ma K, Du J, Zhou Y, Wu G, Qiao X, et al. Characterization of human origin *Salmonella* Serovar 1,4,[5],12:i:- in Eastern China, 2014 to 2018. *Foodborne Pathog Dis.* (2021) 18:790–7. doi: 10.1089/fpd.2021.0008

53. Betancor L, Yim L, Martínez A, Fookes M, Sasias S, Schelotto F, et al. Genomic comparison of the closely related *Salmonella* enterica *Serovars Enteritidis* and Dublin. *Open Microbiol J.* (2012) 6:5–13. doi: 10.2174/1874285801206010005

frontiers | Frontiers in Medicine

TYPE Review
PUBLISHED 05 May 2023
DOI 10.3389/fmed.2023.1155751

OPEN ACCESS

EDITED BY
Milad Badri,
Qazvin University of Medical Sciences, Iran

REVIEWED BY
Farhad Nikkhahi,
Qazvin University of Medical Sciences, Iran
Aida Vafae Eslahi,
Tarbiat Modares University, Iran

*CORRESPONDENCE
Miguel O'Ryan
✉ moryan@uchile.cl

†These authors have contributed equally to this work and share first authorship

RECEIVED 31 January 2023
ACCEPTED 14 April 2023
PUBLISHED 05 May 2023

CITATION
Montero DA, Vidal RM, Velasco J, George S, Lucero Y, Gómez LA, Carreño LJ, García-Betancourt R and O'Ryan M (2023) *Vibrio cholerae*, classification, pathogenesis, immune response, and trends in vaccine development.
Front. Med. 10:1155751.
doi: 10.3389/fmed.2023.1155751

Vibrio cholerae, classification, pathogenesis, immune response, and trends in vaccine development

David A. Montero[1†], Roberto M. Vidal[2,3†], Juliana Velasco[4,5], Sergio George[2], Yalda Lucero[2,6], Leonardo A. Gómez[1], Leandro J. Carreño[3,7], Richard García-Betancourt[7] and Miguel O'Ryan[2*]

[1]Departamento de Microbiología, Facultad de Ciencias Biológicas, Universidad de Concepción, Concepción, Chile, [2]Programa de Microbiología y Micología, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Santiago, Chile, [3]Instituto Milenio de Inmunología e Inmunoterapia, Facultad de Medicina, Universidad de Chile, Santiago, Chile, [4]Unidad de Paciente Crítico, Clínica Hospital del Profesor, Santiago, Chile, [5]Programa de Formación de Especialista en Medicina de Urgencia, Universidad Andrés Bello, Santiago, Chile, [6]Departamento de Pediatría y Cirugía Infantil, Hospital Dr. Roberto del Rio, Facultad de Medicina, Universidad de Chile, Santiago, Chile, [7]Programa de Inmunología, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Santiago, Chile

Vibrio cholerae is the causative agent of cholera, a highly contagious diarrheal disease affecting millions worldwide each year. Cholera is a major public health problem, primarily in countries with poor sanitary conditions and regions affected by natural disasters, where access to safe drinking water is limited. In this narrative review, we aim to summarize the current understanding of the evolution of virulence and pathogenesis of *V. cholerae* as well as provide an overview of the immune response against this pathogen. We highlight that *V. cholerae* has a remarkable ability to adapt and evolve, which is a global concern because it increases the risk of cholera outbreaks and the spread of the disease to new regions, making its control even more challenging. Furthermore, we show that this pathogen expresses several virulence factors enabling it to efficiently colonize the human intestine and cause cholera. A cumulative body of work also shows that *V. cholerae* infection triggers an inflammatory response that influences the development of immune memory against cholera. Lastly, we reviewed the status of licensed cholera vaccines, those undergoing clinical evaluation, and recent progress in developing next-generation vaccines. This review offers a comprehensive view of *V. cholerae* and identifies knowledge gaps that must be addressed to develop more effective cholera vaccines.

KEYWORDS

***Vibrio cholerae*, cholera toxin, cholera, diarrhea, oral vaccine, next-generation vaccines**

1. Introduction

Cholera is an acute, watery diarrheal disease caused by *Vibrio cholerae*, a curved, rod-shaped, motile, Gram-negative bacterium that lives in aquatic environments. Without prompt treatment, cholera can cause severe dehydration and death. Treatment involves administering saline oral rehydration solutions, intravenous fluids, or antibiotics, depending on the severity (1–3).

V. cholerae is spread from person to person via the fecal-oral route or indirectly through contaminated food and water (3). Cholera is endemic in many regions of Africa and Asia, where seasonal or sporadic outbreaks occur (4–7), predominantly in countries with poor sanitary conditions, such as open defecation, unhygienic food handling, and limited access to safe drinking water (8).

Vibrio cholerae is of major public health concern due to its potential to cause pandemics. Since 1817, there have been seven cholera pandemics, with the seventh beginning in 1961 and continuing until today. In 2015, the estimated annual incidence of cholera was 1.3–4 million cases, resulting in 21,000–143,000 deaths (9). However, the notification of cholera cases to the WHO is not mandatory; therefore, it is an underreported disease in many countries (9). For several reasons, the true burden of cholera is underestimated. For instance, it is often difficult to differentiate cholera from other acute diarrheal diseases based on clinical observation. Additionally, diagnostic and epidemiological surveillance laboratories may be deficient or even absent in cholera-endemic areas, thereby limiting accurate etiological diagnosis. It is likely that many cholera-associated cases and deaths do not present to health facilities and are therefore not included in the reports. Added to this, in some countries, there might be disincentives to report cases due to the possible negative impact on tourism and the export industry (10). Recently, the SARS-CoV-2 pandemic has affected cholera surveillance in many regions (11, 12), and there were 65% fewer cases reported to the WHO in 2020 than in 2019 (13). At the same time, preventive measures implemented during the pandemic, such as handwashing, hygiene promotion, social distancing, and banning of large gatherings, likely reduced cholera transmission. The extent to which the SARS-CoV-2 pandemic affected cholera surveillance and epidemiology is currently unknown (14, 15). Thus, cholera remains a leading cause of morbidity and mortality in several developing and resource-poor countries (14).

Cholera is a preventable and treatable disease, and several strategies can be used to control it (Box 1). In 2017, the Global Task Force for Cholera Control proposed an ambitious plan to eliminate endemic cholera in 20 countries and reduce cholera deaths by 90% by 2030 (22). The plan, called "Ending Cholera: A Global Roadmap to 2030," focuses on strengthening public health systems, improving surveillance for early detection of cholera outbreaks, improving drinking water, sanitation, and hygiene conditions, making oral rehydration treatments more accessible, and increasing vaccination coverage.

Antibiotic prophylaxis can theoretically prevent both human-to-human and environment-to-human cholera transmissions. Also, some field trials have suggested that chemoprophylaxis has a protective effect among household contacts of people with cholera (23, 24). However, due to the risk of resistance selection, antibiotic prophylaxis for close contacts, as well as for travelers arriving in or departing from cholera-affected areas, is not usually recommended (25).

Efforts and research directed toward the development of cholera vaccines date back more than a century. The first cholera vaccine, a live whole-cell injectable formulation, was developed in 1885 (26). A few years later, killed and attenuated cholera vaccines were reported in 1888 and 1892, respectively (27). Other injectables cholera vaccines were developed throughout the first half of the 20th century. However, all these vaccines had low levels of protective efficacy (PE) and a concerning history of adverse effects (28).

The start of the seventh cholera pandemic in the 1960s and the spread of this disease throughout Asia and Africa led to increased international interest and funding for cholera research, resulting in the development of the first oral cholera vaccine (OCV). It should be noted that current OCVs exhibit variable PE in human populations for several reasons, including the presence of different *V. cholerae* strains in endemic areas, immunization coverage, malnutrition, co-infections, and variations in the gut microbiome (29, 30). Thus, a cholera vaccine that provides broad and long-lasting protection remains elusive.

In this review, we will discuss recent advances in understanding the *V. cholerae* pathogenesis and immunity against cholera, as well as the current status of approved cholera vaccines. Lastly, we discuss how all this knowledge gained could lead to the development of next-generation cholera vaccines.

2. *Vibrio cholerae* classification

Vibrio cholerae is divided into more than 200 serogroups determined by the structure of the O-antigen of lipopolysaccharide (LPS) (Figure 1A). Among them, a subset of strains belonging to serogroups O1 and O139 can cause cholera and epidemics due to their ability to produce cholera toxin (CTX). Serogroups that are not O1

BOX 1 Cholera prevention and control strategies.

- **Improved sanitation and access to drinking water**: This disease is primarily spread through the consumption of contaminated water or food. Therefore, improving access to drinking water and sanitation facilities can contribute to reducing the risk of cholera transmission (16).
- **Early detection and prompt treatment**: Rapid detection of cholera cases and adequate treatment can reduce the spread of the disease and decrease the number of deaths. Rapid diagnostic tests are useful in this regard (17).
- **Vaccination**: Oral cholera vaccines (OCVs) have been shown to be effective in preventing cholera and should be used as part of a comprehensive cholera control strategy, especially in endemic areas or during outbreaks (18).
- **Health education**: Education campaigns can help to raise awareness about cholera and how to prevent it. These campaigns should include information on proper food storage and preparation, hand washing, and recognizing the signs and symptoms of cholera (19).
- **Strengthening health systems**: A strong health system is crucial for effective prevention, detection, and response to cholera. This requires trained health workers, laboratory capacity, and adequate supplies of vaccines, antibiotics, and oral rehydration solutions (20).
- **Antimicrobial resistance (AMR) surveillance**: Severe cholera is treated with antibiotics, but the emergence of antibiotic-resistant strains can make treatment more difficult. AMR surveillance is essential to ensure appropriate antibiotic use and prevent the spread of resistant strains (21).
- **International cooperation**: Cholera is a global health problem and requires a coordinated global effort. The WHO, along with non-governmental organizations (NGOs) and other international organizations, plays a key role in coordinating efforts to control cholera.

While these strategies can help to control the burden of cholera and prevent large outbreaks, it is important to note that *V. cholerae* will likely never be completely eradicated, as this bacterium is ubiquitous in aquatic environments.

and O139, collectively referred to as non-O1/non-O139, typically lack the CTX and cause small gastroenteritis outbreaks, sporadic cases of bacteremia, and wound infections, but they do not cause cholera (31–33). Unlike O1, more than 85% of non-O1 serogroups (including O139) have a capsule that is critical for virulence in extraintestinal infections (34).

Furthermore, O1 strains are divided into three serotypes, designated Ogawa, Inaba, and Hikojima, which are grouped according to the methylation status of the terminal perosamine of the LPS. Ogawa strains are methylated, Inaba strains are unmethylated, and Hikojima strains express both methylated and unmethylated O-antigens. While the Ogawa and Inaba serotypes can co-circulate during epidemics and are capable of interconverting (35), the Hikojima serotype is rare, and evidence indicates that it is an unstable transitional form that results when a strain undergoes serotype switching from Ogawa to Inaba (36).

Biotype is another key classifier of *V. cholerae* O1 strains. Classical and El Tor biotypes can be distinguished according to a set of phenotypic and genetic markers (37, 38). Interestingly, there are some differences in the infection patterns between both biotypes. El Tor strains are more efficient at host-to-host transmission, survive better in the environment and the human gut, and have a higher occurrence of asymptomatic than symptomatic carriers, compared to the Classical strains (39).

3. Cholera epidemics and pandemics

It seems that the first five cholera pandemics were caused by Classical biotype strains (1817–1896) (Figure 1B) (40). After this, the sixth cholera pandemic (1899–1923) was caused by the Classical biotype. The Classical biotype was prevalent until the 1960s, but during the pre-seventh-pandemic period (1923–1961), some sporadic outbreaks associated with the El Tor biotype were reported. The ongoing seventh pandemic (1961 to date) is caused by the El Tor biotype (41). Notably, after the emergence of the El Tor biotype, the Classical biotype declined and disappeared by the 1980s, and it is now considered extinct (42).

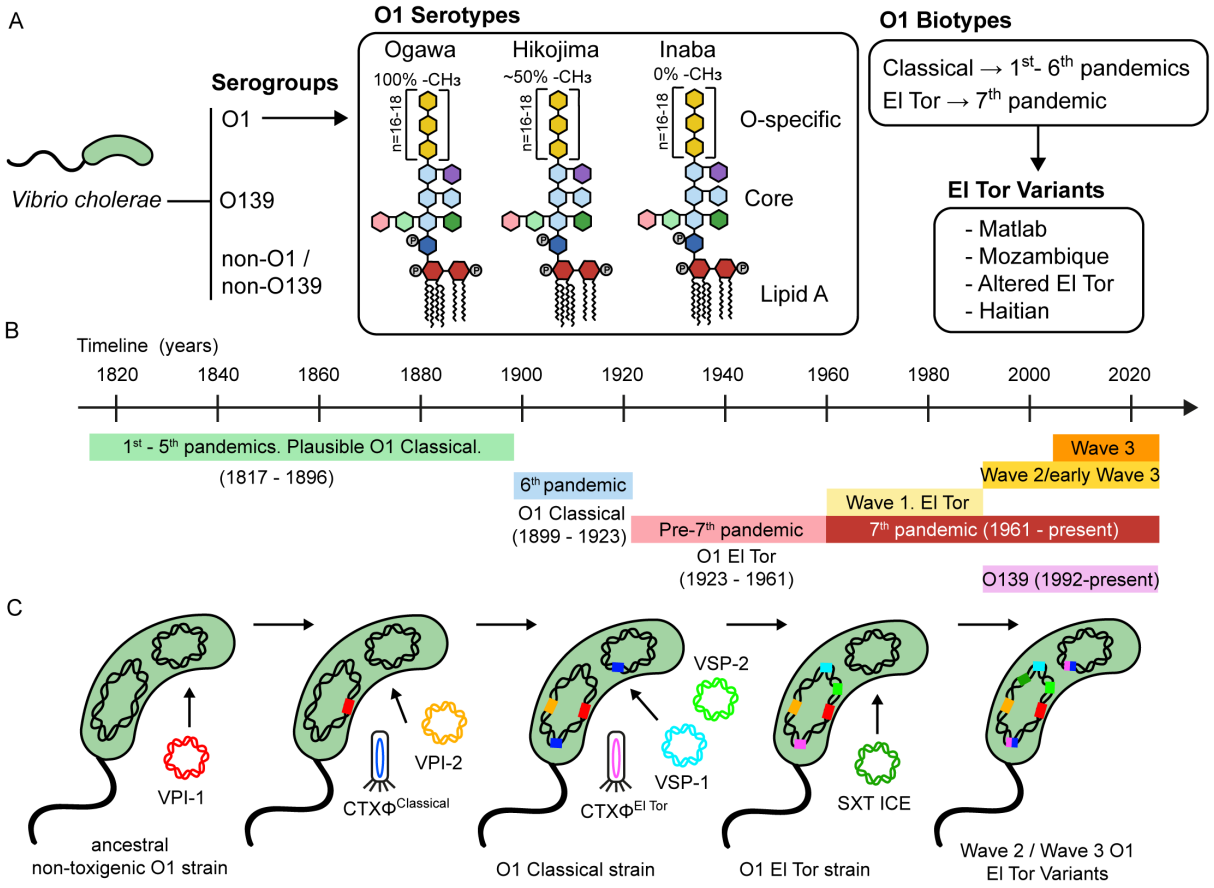

FIGURE 1
Classification and evolution of *V. cholerae*. **(A)** *V. cholerae* is classified into serogroups based on the composition of the O antigen of LPS. Strains belonging to the O1 serogroup are further divided into three serotypes, namely Ogawa, Hikojima, and Inaba. The LPS of these three serotypes is schematically represented, showing the approximate percentage of methylation of the terminal perosamine. Serogroup O1 is also classified into the Classical and El Tor biotypes, based on phenotypic and genetic markers. Over the past two decades, there has been a growing number of reports on *V. cholerae* strains that possess genetic features from both the Classical and El Tor biotypes, leading to the emergence of hybrid or variant strains. These strains have been linked to several cholera outbreaks worldwide and have contributed significantly to the global burden of this disease. **(B)** Timeline of the history of cholera pandemics. **(C)** A schematic representation of the evolutionary process underlying the development of virulence in serogroup O1. This process is mainly driven by the acquisition of mobile genetic elements, including bacteriophages, genomic islands, integrative and conjugative elements, among others.

TABLE 1 Main mobile genetic elements harbored by pandemic *V. cholerae* strains.

Mobile genetic elements	Description
Prophage CTXΦ	It is a filamentous bacteriophage of ~6.7 kb single-stranded DNA that contains the *ctxA* and *ctxB* genes encoding CTX, as well as the *zot and ace* genes encoding accessory toxins (50).
Prophage TLCΦ	It is a satellite bacteriophage of ~5.3 kb in size that facilitates stable integration of CTXΦ (51).
Vibrio pathogenicity island-1 (VPI-1)	Also known as TCP island, it is ~41.3 kb in size. It integrates into the Chr1 and contains genes encoding the toxin-coregulated pilus (TCP), the ToxR regulon, and the metalloprotease TagA (52).
Vibrio pathogenicity island-2 (VPI-2)	It is ~57 kb in size. It integrates into the Chr1 and contains several gene clusters, including genes required for the scavenging (Sialidase, *nanH*), transport (*dctPQM*), and catabolism (*nan-nag* region) of sialic acid (53).
Vibrio seventh pandemic island-1 (VSP-1)	It is ~16 kb in size. It integrates into the Chr1 and encodes the dinucleotide cyclase (DncV) enzyme, which is essential for producing intracellular signaling molecule cAMP- GMP. DncV is required for efficient intestinal colonization of the seventh-pandemic strains (54).
Vibrio seventh pandemic island-2 (VSP-2)	It is ~26.9 kb in size. It Integrates into the Chr1 and encodes RNase H1, DNA repair protein, methyl-accepting chemotaxis proteins, and type IV pilus. VSP-2 could be necessary for the evolutionary fitness and epidemic spread of the seventh pandemic strains (55).
SXT integrative and conjugative element (ICE)	It is ~100 kb in size. It carries multiple antibiotic-resistance genes that confer resistance to sulfamethoxazole, trimethoprim, and streptomycin (56).
Superintegron	Located in the Chr2, it is a large gene capture system of approximately 125 kb, predominantly comprising hypothetical genes, and is proposed as a source of genetic variation (57).

Eight distinct phylogenetic lineages have been identified based on whole-genome sequencing and genomic analyses of different pandemic strains. The L1 and L3-L6 lineages include Classical strains from the first six pandemics. The L2 lineage includes the El Tor strains of the seventh pandemic (7PET) and is subdivided into three clades (waves 1–3) that represent independent waves of transmission (43). Subsequent analysis reported subclades within individual waves and several transmission events, namely, T1-T12 from African countries, LAT-1 to LAT-3 from Latin America, and T13 from East Africa and Yemen (43–45).

Wave 1 strains were prevalent between 1961 and the early 1990s. During the 1990s, serogroup O139 emerged and caused cholera epidemics in Southeast Asia, but its incidence declined a few years later, and it is now rarely isolated. At the same time, Wave 2 and early Wave 3 strains emerged and replaced Wave 1 strains. Interestingly, many Wave 2 and Wave 3 strains display a mix of phenotypic and genotypic traits of Classical and El Tor biotypes, suggesting that they are genetic hybrids (37). These hybrid strains include the Matlab variants from Bangladesh, the Mozambique variants, the Haitian variants, and the altered El Tor biotype from various parts of the world (46). While Wave 2 strains have waned since the 2000s, Wave 3 strains are now the dominant cause of cholera globally (47).

4. Genome and evolution of virulence of *Vibrio cholerae*

The genus *Vibrio* commonly harbors two nonhomologous circular chromosomes, Chr1 and Chr2 (48). The first complete genome sequence of a *V. cholerae* strain was announced for the clinical isolate O1 El Tor Inaba N16961 (49). Genomic analysis of this strain revealed that Chr1 has 2.96 Mb with a 47.7% G + C content, while Chr2 has 1.07 Mb with a 46.9% G + C content. Chr1 contains a large number of genes for essential cellular functions, such as DNA replication, transcription, translation, and cell-wall biosynthesis, as well as virulence genes encoding toxins, adhesins, and surface antigens. By contrast, the Chr2 has fewer such genes and contains a very large integron comprising genes with diverse functions. Comprehensive analysis of both chromosomes revealed the presence of a suite of mobile genetic elements (MGEs), including prophages, genomic islands (GIs), and integrative and conjugative elements (ICEs) (49). Table 1 describes a select list of MGEs that are important in pandemic *V. cholerae* strains.

The genomic plasticity of *V. cholerae* and its ability to exchange genes through natural transformation, conjugation, and transduction are hallmarks of this bacterium. Its evolution is continuous due to the acquisition or loss of genomic segments (58, 59). The acquisition of MGEs is known to be the major driver for the evolution of *V. cholerae* virulence and a determinant of genetic divergence between environmental and pandemic strains (60, 61). In this respect, understanding the evolutionary events that lead to the emergence of pandemic clones of *V. cholerae* might provide new approaches for controlling this pathogen.

Chun et al. (62) proposed a hypothetical evolutionary pathway for the emergence of the seventh pandemic *V. cholerae* strains (Figure 1C). According to this model, the diversification of a common ancestral strain occurred through the sequential acquisition of MGEs, likely driven by environmental factors. After acquiring the O1 antigen, an O1 progenitor strain probably acquired the *Vibrio* pathogenicity island-1 (VPI-1) and *Vibrio* pathogenicity island-2 (VPI-2), which are ubiquitous among strains from the sixth (Classical biotype) and seventh (El Tor biotype) pandemics (63). VPI-1 encodes the toxin-coregulated pilus (TCP), which is the receptor for bacteriophage CTXΦ. Thus, transduction by the CTXΦ must have been preceded by the acquisition of VPI-1. The divergence between the Classical and El Tor biotypes was due to the acquisition of distinct bacteriophages CTXΦ and the *Vibrio* seventh pandemic islands (VSP-1 and VSP-2). Several lines of evidence support this. For example, comparative nucleotide sequence analyses have revealed that the CTXΦ from Classical and El Tor biotypes comprise two distinct lineages, indicating

that they were acquired in independent events (64–66). In addition, VSP-1 and VSP-2 are consistently found in the O1 El Tor and O139 strains but are predominantly absent in the O1 Classical strains isolated between 1817 and 1923 (40, 59).

Horizontal gene transfer events have also occurred among strains from the seventh pandemic. Unlike Wave 1 strains, Wave 2 and Wave 3 strains contain a self-transmissible integrative conjugative element that carries multiple antibiotic-resistance genes (SXT ICE). The acquisition of SXT ICE likely influenced the population shift from the Wave 1 to Wave 2/3 strains (43). Interestingly, O139 strains that emerged in the 1990s also harbor the SXT ICE (56). In addition, Wave 2 and Wave 3 strains have undergone multiple CTXΦ substitutions and replacements, leading to the emergence of El Tor variant strains (47, 67).

5. Pathogenesis of *Vibrio cholerae*

In this section, we will review the current understanding of the pathogenesis of toxigenic *V. cholerae* strains, particularly the O1 serogroup. Much of this information has been obtained from *in vitro* assays and challenge experiments in animal models, although some findings have been subsequently confirmed in human infections. Table 2 provides a summary of the main virulence factors of *V. cholerae* that are expressed during infection, and Figure 2A depicts some of these virulence factors.

The incubation period of cholera can range from 12 h to 5 days (106, 107). Once ingested, *V. cholerae* must rapidly adapt to the human digestive system (Figure 2B). To accomplish this, the bacterium uses a complex signal transduction network that regulates gene expression in response to different environments and stimuli throughout the gastrointestinal tract.

Vibrio cholerae is highly sensitive to low pH, and during passage through the stomach, the vibrios undergo an acid tolerance response (ATR) to both inorganic and organic acid. ATR involves several proteins, including the porin OmpU and the transcriptional regulators CadC and HepA, among others (108–110). Despite the ATR, the number of vibrios reaching the small intestine is reduced. In fact, a high infectious dose (10^8 bacteria) is required to cause severe cholera in healthy volunteers, while a lower dose (10^5 bacteria) is sufficient when given with antacids to neutralize stomach acid (28, 111).

Upon reaching the small intestine, the main site of colonization, adaptation to antimicrobial agents, such as bile salts and antimicrobial peptides, is crucial. To achieve this, the bacterium modulates its outer membrane protein (OMP) profile through the activation of a tightly regulated signaling pathway known as the ToxR Regulon (112). In the presence of bile salts, ToxR upregulates the expression of OmpU and downregulates the expression of OmpT, two of the most abundant OMPs of *V. cholerae* (113). The change of the OMP composition also involves removal of OmpT by outer membrane vesicle (OMV) production (114). OmpU and OmpT have distinct channel properties: OmpU is more cation-selective than OmpT, and the bile salt deoxycholic acid blocks OmpT porin activity but not that of OmpU (115, 116). Therefore, OmpU confers resistance to bile salts and antimicrobial peptides, playing a crucial role in the colonization and survival of *V. cholerae* in the small intestine (87, 88). Other intestinal environmental signals, such as bicarbonate, mucin, and osmolarity, also modulate the expression of virulence factors in *V. cholerae* (117–120).

To successfully colonize the small intestine, *V. cholerae* must penetrate a highly viscous mucus layer that is approximately 100–400 μm thick (121), or roughly 30–130 times the size of the bacterium. For this, the vibrios use their flagellum to propel through the mucus layer and reach the epithelial surface (122). It is worth noting that nonmotile vibrios are significantly less efficient at colonization or even avirulent (84). Additionally, the penetration of the mucus layer is facilitated by the hydrolysis of mucins by a group of enzymes, such as HapA, TagA, among others (91, 93–95, 123). Vibrios that fail to penetrate the mucus layer do not colonize the intestinal mucosa and are shed in the feces due to the continuous production and replenishment of mucus (124).

Meanwhile, *V. cholerae* needs to overcome host immunity (see next section) and the colonization resistance mechanisms of the gut microbiota (125). In this respect, mucin activates the *V. cholerae* type VI secretion system (T6SS), which operates as a molecular syringe that kills bacterial competitors through the contact-dependent translocation of toxic effectors (104, 126). In mice, *V. cholerae* T6SS has been shown to attack members of the host commensal microbiota, facilitating intestinal colonization (105). Moreover, T6SS has been suggested as a key mechanism conferring enhanced fitness to pandemic *V. cholerae* strains (127). However, secondary bile acids generated by gut microbiota can inhibit the assembly of the T6SS apparatus (126). Recently, differences in the gut microbiota among individuals have been suggested as a possible explanation for the susceptibility or resistance to cholera (125, 128).

The initial attachment of *V. cholerae* to intestinal epithelial cells (IECs) is likely mediated by the GbpA protein. GbpA is regulated by quorum sensing and is expressed at low cell density (129). Additionally, GbpA stimulates mucin secretion by IECs, which in turn enhances GbpA expression (130). GbpA has been shown to bind mucin, and deletion of its encoding gene decreases intestinal colonization in the infant mouse model (100, 101, 130). Other adhesive factors that could play a role in attachment to the intestinal epithelium are the OmpU and FrhA proteins (90, 102, 131).

After attachment to the intestinal epithelium, *V. cholerae* decreases its motility, proliferates, and forms microcolonies, mostly originating from single vibrio cells (95). Colonizing vibrios express CTX and toxin-coregulated pilus (TCP), which are their main virulence factors. CTX is responsible for the secretory diarrhea characteristic of cholera, while TCP mediates adherence and microcolony formation. Both acidic bile and bicarbonate have been shown to induce CTX and TCP expression via the ToxR regulon (112, 119, 132). Importantly, TCP-deficient mutant strains are unable to colonize animal models and the human intestine (133–135).

CTX is secreted into the extracellular milieu through the type II secretion system (T2SS) (69). Then, the cellular uptake of CTX occurs via endocytosis, mediated by the binding of CTX-B pentamer to GM1 ganglioside receptors located on the surface of IECs (Figure 3). Of note, NanH cleaves sialic acid from high order gangliosides to release sialic acid and expose the GM1 ganglioside (96, 97). Therefore, NanH promotes the internalization of CTX and its toxigenic effects (139). Although GM1 is considered the primary receptor of CTX, recent studies suggest that CTX-B also binds histo-blood group antigens (HBGAs) at a secondary binding site (140). Additionally, CTX can be released as cargo inside OMVs, which protects the toxin from degradation by intestinal proteases, potentially preserving its toxic effects for longer periods of time (70–72). In particular, CTX-containing OMVs have been shown to be internalized by

TABLE 2 Selected virulence factors of *V. cholerae* expressed during human infection.

Virulence factor	Description
Main virulence factors	
Cholera toxin (CTX)	CTX is the main virulence factor in toxigenic *V. cholerae* strains. It belongs to the AB5 family of toxins, which are composed of the catalytic A subunit (CTX-A) and the pentameric receptor-binding B subunit (CTX-B). These subunits are encoded by the *ctxA* and *ctxB* genes located in the filamentous bacteriophage CTXϕ (68). CTX is responsible for the secretory diarrhea characteristic of cholera. It is secreted through the type II secretion system (T2SS) and as cargo within outer membrane vesicles (OMVs) (69–72).
Toxin-coregulated pilus (TCP)	TCP is a type IV pilus with structural similarities to the T2SS. Bacterial aggregation in the form of microcolonies through pilus-pilus interaction with TCP is required to colonize the human intestine. The expression of TCP is coordinately upregulated with that of CTX (73). Furthermore, TCP is the receptor for CTXΦ. Therefore, the evolution of virulence in non-toxigenic *V. cholerae* strains involves the sequential acquisition of VPI followed by CTXΦ (52).
Accessory toxins	
Multifunctional autoprocessing repeats-in-toxin (MARTX) toxin	MARTX toxin is secreted through the type I secretion systems (T1SS). This toxin forms pores in the membranes of target eukaryotic cells and translocates multiple functionally independent effector domains, each of which disrupts a key cellular process. This toxin disrupts the actin cytoskeleton, inhibits phagocytosis, and suppresses innate immune signaling in intestinal epithelial cells (IECs), preventing neutrophil recruitment and bacterial clearance (74, 75).
Hemolysin A (HlyA)	HlyA, also known as Cytolysin (VCC), is a toxin that exhibits vacuolizing and pore-forming activity, resulting in ion leakage and eventual cellular death (76, 77). It is secreted through the T2SS as an inactive 79-kDa pro-hemolysin and undergoes post-translational N- terminal cleavage, mainly mediated by the HapA protease, to form an active 65-kDa toxin (78, 79). HlyA is also secreted in association with OMVs (79). Deletion of the *hlyA* gene reduces virulence in infant mice but has no impact on the rate of mild diarrhea in humans (80, 81).
Zonula occludens toxin (Zot)	It affects the structure of actin microfilaments, leading to increased permeability of epithelial tight junctions (TJ), resulting in the passage of large molecules through a paracellular route (82).
Accessory cholera enterotoxin (Ace)	Ace is an integral membrane protein that alters ion transport, causes accumulation in ligated rabbit ileal loops, and is responsible for mild diarrhea. Ace may cause initial intestinal secretion before CTX acts by stimulating Ca^{2+} – dependent Cl^{-} /$HCO3^{-}$ symporters causing extracellular Ca^{2+} influx (83).
Virulence factors associated with intestinal colonization	
Flagella	*V. cholerae* has a single polar flagellum that is used to penetrate the mucin layer; non-motile (aflagellated) vibrios are significantly less efficient at adhesion and colonization or even avirulent (84–86).
Outer membrane protein U (OmpU)	OmpU confers resistance to bile salts and antimicrobial peptides, playing a key role in the survival of *V. cholerae* in the human intestine (87–89). Moreover, OmpU could play a role in adhesion to the intestinal epithelium (90).
Haemagglutinin/protease (HapA)	HapA is a Zn-dependent metalloprotease secreted through the T2SS as a free protease or in a cell-associated form (73). HapA exhibits several proteolytic activities, including modifying toxins and degrading mucin, fibronectin, and lactoferrin (91). It also acts on TJ-associated proteins, disrupting the paracellular barrier function (92). HapA promotes penetration of the mucosal layer, as well as detachment and spreading of infection along the gastrointestinal tract (93).
ToxR-activated gene- A (TagA)	TagA is a 115 kDa secreted metalloprotease that cleaves mucin glycoproteins and cell-surface glycans, which *V. cholerae* could use as a source of nutrients (94, 95).
Sialidase (NanH)	NanH, also known as neuraminidase, is an extracellular enzyme that catalyzes the cleavage of terminal sialic acid residues from complex carbohydrates on glycoproteins and glycolipids. It is secreted through the T2SS (78). NanH specifically removes sialic acid residues from higher-order gangliosides on the membranes of IECs, exposing GM1 gangliosides, the binding site for CTX (96, 97). Some studies suggest that NanH could promote intestinal colonization as sialic acid residues serve as carbon and energy sources for *V. cholerae* (98).
GlcNAc-binding protein (GbpA)	GbpA is secreted through the T2SS (99). It facilitates attachment to the chitinous exoskeleton of zooplankton as well as mucins covering intestinal epithelial cells. Deleting the *gbpA* gene has been shown to affect intestinal colonization in the infant mouse model (100, 101).
Flagellum-regulated hemagglutinin A (FrhA)	FrhA is a large protein (2,251 amino acids) that contains a type I secretion motif and an RTX-like repeat region at the C-terminus. It mediates binding to erythrocytes, epithelial cells, and chitin and enhances biofilm formation. Deletion of the *frhA* gene affects intestinal colonization in the infant mouse model (102).
Secretion systems	
Type I secretion system (T1SS)	Gram-negative bacteria use the T1SS to secrete proteins in a one-step process using ATP. In *V. cholerae*, T1SS is associated with the secretion of RTX proteins such as MARTX (73).

(Continued)

TABLE 2 (Continued)

Virulence factor	Description
Type II secretion system (T2SS)	The T2SS shares many structural characteristics with the type IV pilus. Proteins secreted by the T2SS are first translocated to the periplasm by Sec or Tat, where they are assembled to acquire a secretion-competent conformation. *V. cholerae* uses the T2SS to export more than 20 proteins involved in colonization, biofilm formation, and virulence (73, 103). Deletion of TS22 in *V. cholerae* affects growth, biofilm formation, antimicrobial resistance, and cell envelope integrity (73).
Type VI secretion system (T6SS)	The T6SS is a contractile nanomachine resembling a T4 bacteriophage that kills target cells through the contact-dependent translocation of toxic effectors (104). During experimental infection in mice, *V. cholerae* has been found to use T6SS to attack members of the gut microbiota, thereby facilitating colonization (105).

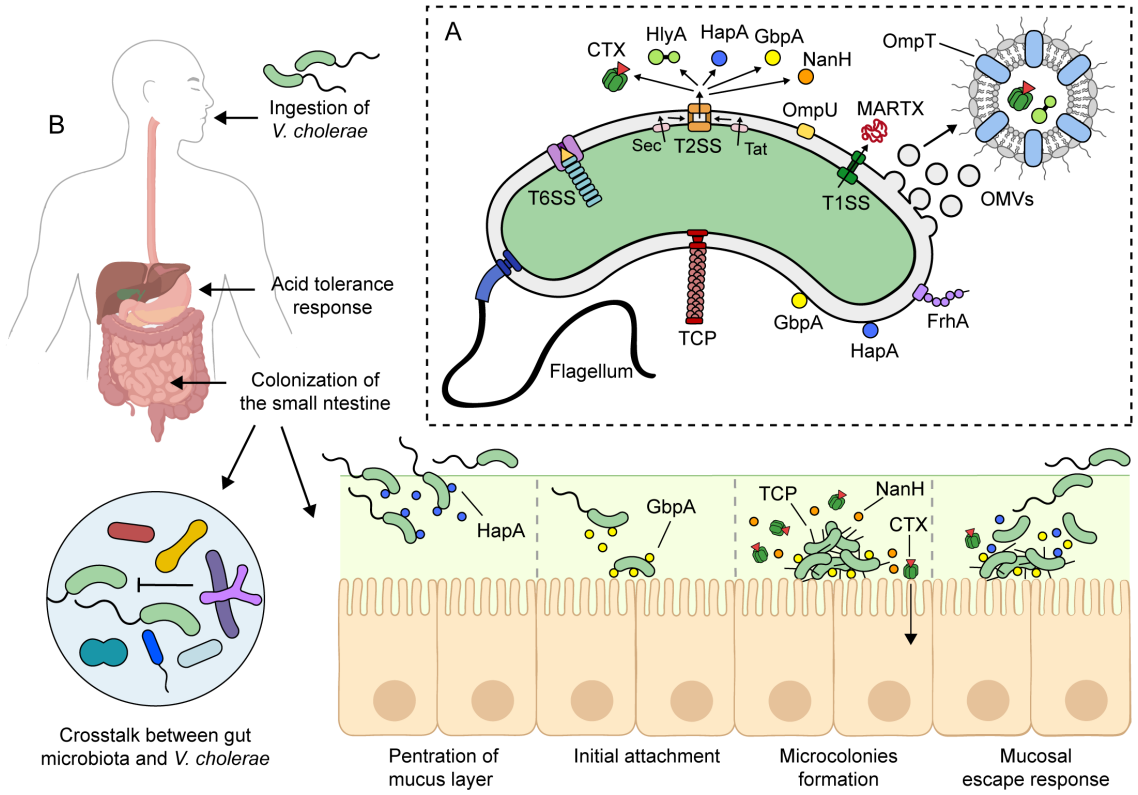

FIGURE 2
Pathogenesis of toxigenic *V. cholerae*. **(A)** Toxigenic *V. cholerae* produces several virulence factors that contribute to its pathogenesis. The precise pathogenic mechanism is not yet fully understood, but it is widely accepted that it involves the combination of these virulence factors and the ability to colonize and persist in the small intestine. **(B)** Upon ingestion, *V. cholerae* survives the low pH of the stomach *via* an acid tolerance response. In the small intestine, *V. cholerae* uses its flagellum to propel through the mucus layer and reach the epithelial surface. Meanwhile, *V. cholerae* must overcome host immunity and the colonization resistance mechanisms of the gut microbiota. To colonize the small intestine, it expresses virulence factors such as toxin-coregulated pilus (TCP) and cholera toxin (CTX). During infection, other factors such as HapA, GbpA, and NanH are also expressed. For more details on the roles of these virulence factors, please refer to the text. This figure was created using BioRender.com.

caveolin-mediated endocytosis in a GM1-independent mechanism that appears to require the presence of OmpU on the vesicle surface (71). After CTX is internalized, cAMP signaling in the IECs is impaired, resulting in a massive release of electrolytes and water into the intestinal lumen, leading to diarrhea (137). The mechanism of action of CTX is described in detail in Figure 3C. Furthermore, other accessory toxins produced by this pathogen can contribute to impaired epithelial barrier function and the development of diarrhea (141). Although 90–95% of infected individuals remain asymptomatic or experience mild symptoms, the remaining 10% develop severe cholera, characterized by profuse watery diarrhea (25). This diarrhea is often described as "rice-water stool" due to its pale, milky appearance (28).

In the late phase of infection, microcolonies of vibrios reach a high cell density, and the nutrients in the intestine decrease. Consequently, vibrios switch from rapid replication to bacteriostasis and downregulate the expression of major virulence factors. Some of them become motile and detach from the epithelial surface moving

into the luminal fluid. This process, known as the "mucosal escape response," is dependent on the general stress response regulator RpoS and the quorum sensing regulator HapR (142–145). Moreover, detachment of vibrios from the intestinal cells is facilitated by the HapA protease, which degrades the GbpA adhesin (129).

Lastly, individuals without effective antibiotic treatment may shed vibrios in their feces for up to 10 days after infection, releasing the bacteria into the environment and increasing the risk of further infections (25). Interestingly, vibrios shed in rice water stool are in a hyperinfectious state (146). These hyperinfectious vibrios are flagellated and highly motile, but most known virulence genes, including those for CTX and TCP, as well as those associated with chemotaxis, are downregulated (147). The exact mechanism for the regulation of the hyperinfectious state remains unknown. In any case, hyperinfectivity is a transient state and is maintained only for a few hours after shedding from the patients (148). Thus, the hyperinfectious state could play a role in the spread of cholera when transmission to another person occurs in a relatively short period of time (149). It is also worth noting that asymptomatic individuals (healthy carriers) are mostly short-term carriers and short-term shedders of vibrios but play an important role in the persistence and transmission of the disease (150).

6. Immune response to cholera

Numerous experimental and epidemiological studies have documented that *V. cholerae* infection induces protection against reinfection for at least 3 years in most patients who recover. In this respect, cholera confers greater protection than a subclinical infection (151). However, several factors can affect the immune response against *V. cholerae* and the consequent establishment of immunological memory, including age, nutritional status, blood group, endemicity, co-infections, microbiota, and others (152).

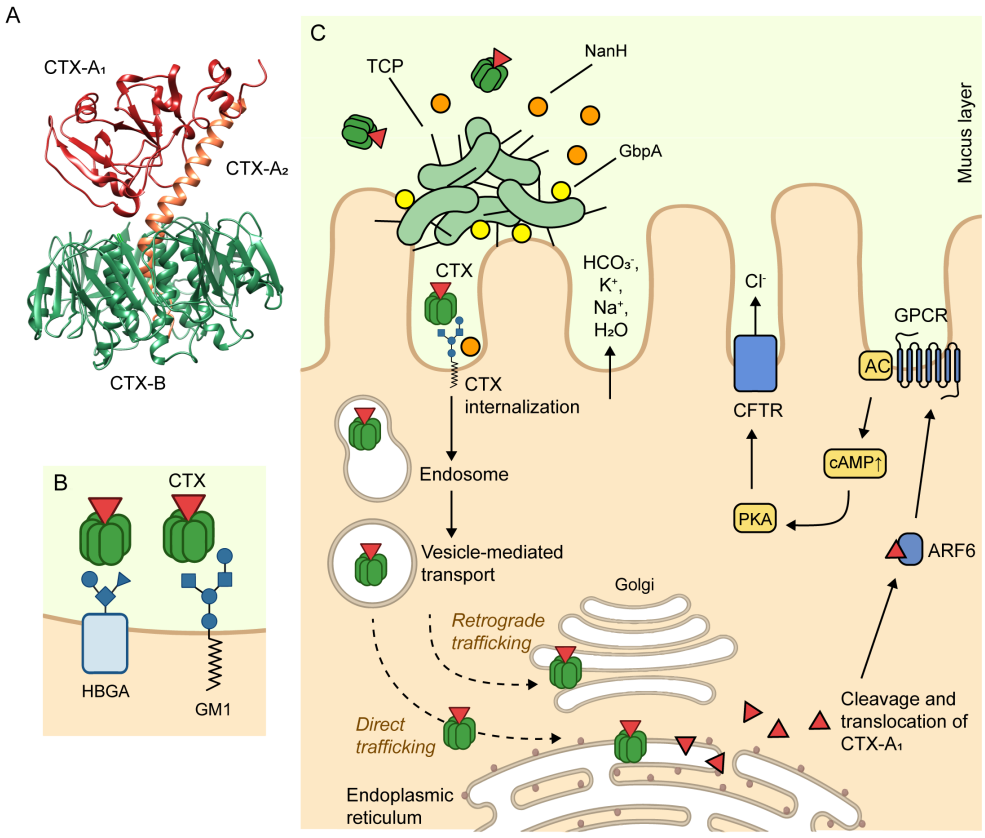

FIGURE 3
Mechanism of action of cholera toxin. **(A)** The crystal structure of CTX (PDB accession number 1XTC) was determined by Zhang et al. (136). CTX is composed of a heterodimeric CTX-A subunit, which consists of two polypeptide chains, CTX-A_1 (22kDa) and CTX-A_2 (5kDa), linked by a single disulfide bond. The CTX-A_2 helical peptide links the CTX-A1 chain to the pentameric CTX-B subunit, which is composed of five identical polypeptide chains (11.6kDa). **(B)** The CTX-B pentamer specifically binds to GM1 gangliosides (primary receptor) or histo-blood group antigens (HBGAs; secondary binding site) present on the apical side of intestinal epithelial cells, promoting the endocytosis of the toxin. **(C)** The internalization of CTX may occur through clathrin-dependent as well as caveolae- and clathrin-independent endocytosis. Regardless of the mechanism of endocytosis, the CTX is internalized to the early endosomal compartment, trafficked to the Golgi, and then onto the endoplasmic reticulum (ER), where it dissociates into a CTX-A_1 and a CTX-A_2/CTX-B complex. Next, the CTX-A_1 is exported out of the ER to the cytosol, where it is activated by ADP ribosylation factor 6 (ARF6). The ARF6-bound, activated CT-A_1 subunit, in turn, activates adenylyl cyclase (AC) by catalyzing ADP ribosylation of a G protein-coupled receptor (GPCR). The AC then catalyzes the conversion of ATP to cyclic adenosine monophosphate (cAMP), increasing the intracellular cAMP concentration. This leads to the activation of protein kinase A (PKA), which phosphorylates the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel proteins, ultimately resulting in the release of electrolytes (Cl^-, HCO_3^-, Na^+, K^+) and water into the intestinal lumen, causing the secretory diarrhea characteristic of cholera (137, 138). The figure was created with BioRender.com.

Although the exact mechanism behind protective immunity against cholera remains largely unknown, our current understanding of *V. cholerae* pathogenesis offers some insight into how this bacterium interacts with the intestinal mucosa and triggers multiple arms of the immune system (Figure 4).

6.1. Innate immune response

Cholera has traditionally been considered a noninflammatory diarrheal disease; however, this concept has been re-evaluated, and now it is known that some inflammation occurs during the acute phase of the infection, which is followed by a non-inflammatory convalescent phase (153, 154). In fact, patients with cholera in the acute phase exhibit ultrastructural changes in the duodenal mucosa, such as the widening of the intracellular spaces and alterations of the apical junctional complexes. Moreover, these changes correlate with clinical severity and are characterized by the infiltration of innate immune cells, strongly suggesting an inflammatory response at the site of infection (155).

In the small intestine, IECs play a fundamental role in the defense against enteric pathogens. First, IECs constitute a physical barrier that restricts bacteria to the intestinal lumen. Second, they act as sensors to detect pathogen-associated molecular patterns (PAMPs) and release cytokines that recruit immune cells to the site of infection (156). Mechanistically, during the onset of *V. cholerae* infection, several immunogenic components of this pathogen, such as LPS, flagellins, CTX, and OmpU, can act as PAMPs and be recognized by extracellular and intracellular pattern recognition receptors (PRRs). This triggers the activation of central innate immune pathways, including the myeloid differentiation primary response gene 88 (MyD88), mitogen-activated protein kinase (MAPK), and nuclear factor kappa light-chain enhancer of activated B cells (NF-κB), which in turn activate the secretion of several proinflammatory cytokines (154, 157–162).

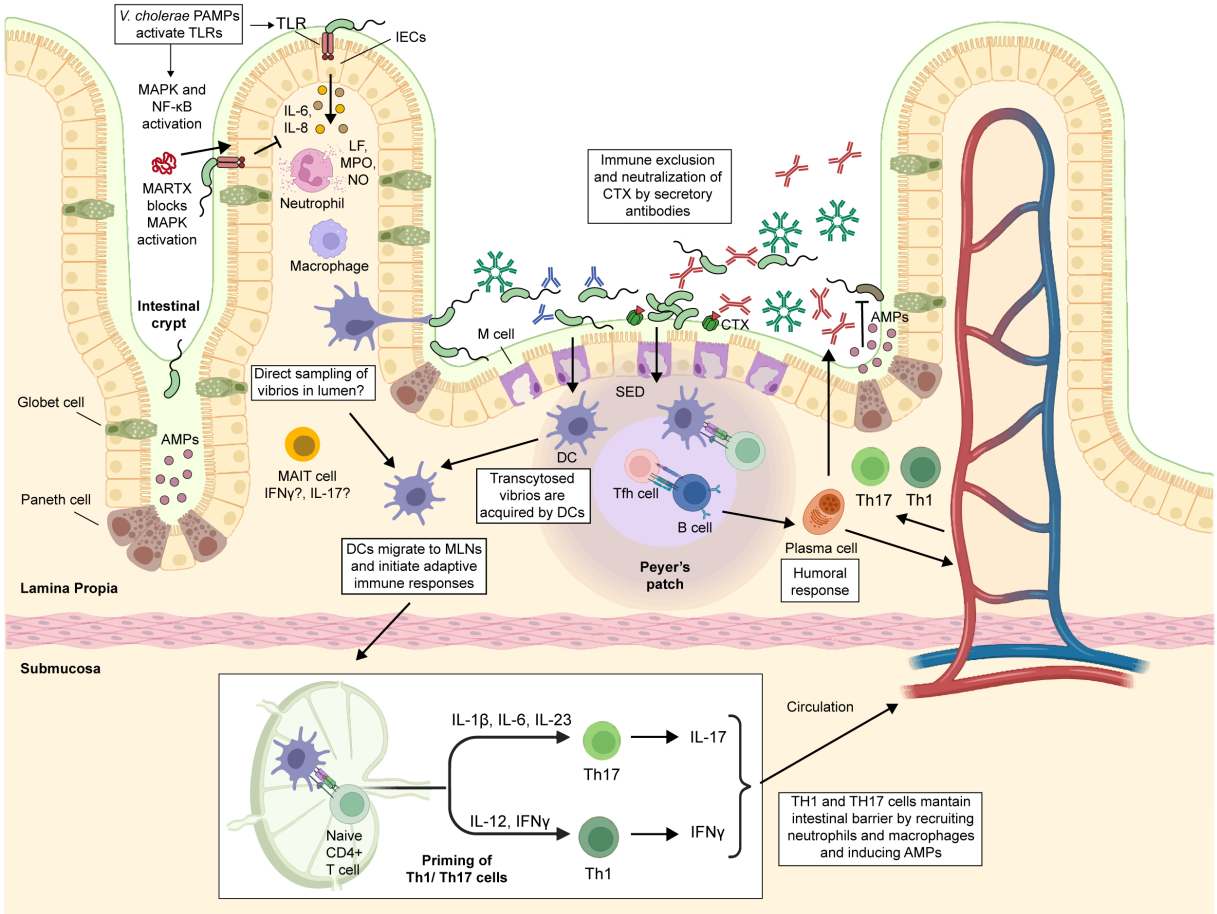

FIGURE 4
Immune response against cholerae infection. Intestinal epithelial cells (IECs) serve as a physical barrier that limits bacteria to the intestinal lumen. They detect PAMPs such as LPS, flagellin, CTX and OmpU, triggering the secretion of proinflammatory cytokines that recruit innate immune cells such as macrophages, dendritic cells (DCs), and neutrophils. Activated neutrophils increase the inflammation of the intestinal lumen through metabolites such as lactoferrin (LF), myeloperoxidase (MPO), and nitric oxide (NO). M cells take up and transport vibrios from the intestinal lumen to the subepithelial dome (SED) region in Peyer's patches, where DCs engulf them. Activated DCs migrate to mesenteric lymph nodes, where they produce Th17 or Th1-driving cytokines. Macrophages can also contribute to Th17 or Th1 differentiation through the secretion of IL-23 and IL-6 or IFNγ, respectively. Th1, Th17, and Tfh cells induce B-cell differentiation and expansion. Mucosal-associated invariant T (MAIT) cells are present and highly activated in the lamina propria of the duodenum of cholera patients, but their exact role in the protection against cholera remains to be determined. Secretory antibodies (sIgA and sIgM) prevent vibrios from attaching to the epithelium, blocking their access to the epithelial surface and facilitating their removal through peristaltic activities. Some IgG antibodies could enter the intestinal lumen *via* passive leakage through a damaged and leaky epithelium. The figure was created with BioRender.com.

For example, *V. cholerae* flagellins induce the expression of IL-1β and IL-8 in IECs by interacting with Toll-like receptor 5 (TLR5) and activating NF-kB and MAPK pathways (159, 161). Likewise, OmpU induces the expression of IL-6, IL-8, and MCP-1 (CCL2) in IECs (163, 164). Moreover, CTX increases intracellular cAMP, leading to IL-6 secretion in IECs (165, 166). These studies were further supported by a transcriptomic analysis of IECs from patients with cholera in the acute phase, where an upregulation of several genes associated with innate immunity was observed (160). Remarkably, activation of the MAPK and NF-κB pathways persisted even 30 days after infection. Furthermore, multiple TLRs, including cell surface TLRs 1, 2, 4, 5, and 6, as well as the endosomal TLRs, including TLR3, TLR7, and TLR8 were upregulated (160).

Among the cytokines mentioned, IL-8 is a potent chemoattractant that recruits polymorphonuclear leukocytes and T cells to the infection site, and stimulates neutrophil degranulation and phagocytosis (167, 168). In addition, the CCL2 chemokine induces the migration of monocytes, dendritic cells, and memory T-cells (169, 170), while IL-6 secretion by IECs activates neutrophil degranulation in the intestinal lumen (166). As a result, innate immune cells, particularly neutrophils, are recruited to the site of infection during the acute phase of cholera (153, 155, 171). Furthermore, a recent study showed that mucosal-associated invariant T (MAIT) cells, an innate-like lymphocyte known to provide immediate effector functions in response to infections, are present and highly activated in the lamina propria of the duodenum of cholera patients (172). Recent evidence suggests that MAIT cells can also provide B cell help and support antibody production at the mucosa level (173); however, further investigation is needed to determine the exact role of MAIT cells in the protection against cholera.

Once neutrophils arrive at the infection site, they express metabolites such as lactoferrin (LF), myeloperoxidase (MPO), and nitric oxide (NO) (153, 174, 175). This may explain the detection of elevated levels of LF and MPO in stools and NO metabolites (NO_2^-/NO_3^-) in serum of patients with cholera during the acute phase (174, 175). Of note, *V. cholerae* is highly susceptible to the bactericidal action of LF (176, 177). By contrast, in the convalescence phase (~ up to 30 days post infection), an increase of mast cells and eosinophils and their respective effector molecules chymase and IL-3 have been reported (153). Consequently, the activation of proinflammatory signaling pathways, the recruitment of innate immune cells, and their effector functions are fundamental in the initial defense against *V. cholerae*.

Despite the above, *V. cholerae* has some strategies to evade the innate immune response of the host (178). A recent study demonstrated that the accessory MARTX (multifunctional-autoprocessing repeats-in-toxin) toxins secreted by some *V. cholerae* strains can block the MAPK signaling pathway in T84 cells grown *in vitro*. Suppression of MAPK signaling in IECs prevented the recruitment of innate immune cells, and thus this mechanism could protect colonizing vibrios from neutrophil-mediated clearance (74). Importantly, the immunomodulatory effect of these toxins may contribute to the differences in inflammation observed between various *V. cholerae* strains (158). In fact, the current predominant circulating altered El Tor strains lack the MARTX toxins due to a stop codon (179), which could explain in part why these hybrid strains cause a more severe diarrhea and increased intestinal inflammation (158). This raises the question: are innate immune responses in cholera beneficial or detrimental to the host? It is likely that adequate tuning of the innate immune system and a subsequent moderate inflammatory response can be protective against severe cholera.

The gut-associated lymphoid tissues (GALT) play a vital role in developing immunity following natural exposure to enteric pathogens (180). GALT is divided into inductive sites, such as the Peyer's patches (PP) and mesenteric lymph nodes (MLNs), and effector sites, such as the lamina propria and the intraepithelial lymphocyte compartment (181). Consequently, upon exposure to *V. cholerae*, protective immunity against this pathogen is expected to largely depend on cellular processes that occur in GALT. In fact, in the rabbit ileal loop model, M cells take up and transport vibrios from the intestinal lumen to the subepithelial dome (SED) region in the PP (182). Thus, it is likely that resident DCs and macrophages in the SED become activated and then phagocytose these vibrios during infection in humans.

CTX induces an increase in intracellular cAMP in innate immune cells, leading to the production of IL-1β, TNF-α, and IL-6 (183). In particular, it has been shown that CTX has an immunomodulatory effect on DCs by stimulating their maturation, as well as the expression of MHC class II and costimulatory molecules (184, 185). CTX activates DCs to produce Th17-driving cytokines, including IL-6, which promotes the differentiation of Th17 cells (186, 187). Significantly, CTX also induces the migration of DCs from the SED region to B and T cell zones, where antigen presentation occurs (188). Therefore, the DCs activated by CTX can induce strong humoral and cellular immunity.

Some insights into the role of macrophages against cholera infection have been obtained using the THP-1 human monocyte-like cell line. THP-1 cells exposed to live toxigenic *V. cholerae* upregulate IL-23 expression (189). In another study, THP-1 cells stimulated with *V. cholerae* LPS exhibited increased expression of TNF-α, IL-1β, and MIP-3α through interaction with TLR4 and subsequent activation of the MyD88 pathway (190). It is important to note that both IL-23 and IL-1β are essential for the differentiation of Th17 cells (191, 192).

6.2. Adaptive immune response

The subsequent adaptive immune response is complex and involves both humoral and cellular mechanisms. In the acute stage of cholera, studies have shown that lamina propria lymphocytes (LPLs) in the duodenum express significant amounts of IL-6, IL-8, IL-12β, and IL-17 cytokines (162, 193). Later, at day 7 post-infection, cholera induces cellular immune responses in blood compatible with Th1 (IFN-γ) and Th17 (IL-17) profiles (193). Additionally, patients who recover from cholera display an increased percentage of gut-homing $CD4^+$ T cells and gut-homing B cells that peak in the circulation 7 days after the infection. Th17 lineage and other IL-17-producing cells play a key role in host defense against bacteria at mucosal surfaces (191), making the Th17 response to *V. cholerae* highly significant. By contrast, the level of gut-homing $CD8^+$ T cells reaches its peak in circulation on day 21 (194, 195).

Cholera also triggers follicular helper T (Tfh) cells, which are essential for germinal center formation, affinity maturation, and the development of most high-affinity antibodies and memory B cells

(196). A recent study demonstrated that cholera infection in the acute phase induces a significant increase in circulating Tfh cells, which impacts the development of antigen-specific B cells and consequent immunoglobulin production (197).

Considering the pathogenesis of *V. cholerae*, a humoral response capable of neutralizing the CTX, blocking bacterial adherence to the mucosa, and opsonizing the bacteria to mediate their clearance is expected. Consistently, patients who recover from cholera develop systemic IgM, IgG, and IgA antibodies, as well as secretory IgA (sIgA) antibodies, which target several antigens, including CTX-A and CTX-B subunits, LPS, O-specific polysaccharide (OSP), LPS, NanH, flagellins (FlaB, FlaC, and FlaD), TcpA, and HlyA (139, 198–204). Nevertheless, while CTX-specific antibodies confer short-term immunity, the antibacterial (vibriocidal) antibodies are associated with protection against colonization and long-term protective immunity (205).

The best-characterized correlate of protection against cholera is the vibriocidal antibody titer (VAT), which measures the minimum concentration of serum required for antibody-dependent complement-mediated bacterial killing (206). However, VAT is not a comprehensive predictor of long-term immunity. For instance, a specific VAT threshold for absolute protection has not been identified; infection still occurs in a few individuals with very high titers (207). Moreover, there is a lack of mechanistic connection between levels of circulating VAT and prevention of *V. cholerae* colonization at the intestinal mucosal surface level. At the same time, anti-body-dependent complement-mediated bacterial killing is relevant for immunity against systemic infections, it appears to be less important at the intestinal mucosa due to low complement levels at this site (206, 208).

The mechanism by which IgG enters the intestinal lumen is unclear, but it may occur via passive leakage through a damaged and leaky epithelium or through FcRn-mediated epithelial transport (209, 210). Notably, recent studies have demonstrated that IgG anti-OSP contributes to protection against cholera by inhibiting the motility of *V. cholerae*, potentially limiting its access to the apical surface of the intestine (211). It is also possible that anti-OSP sIgA may contribute to protection by inhibiting motility and trapping the bacteria at the mucosal surface (1, 212). Even in the absence of circulating anti-OSP antibodies, OSP-specific memory B cells may maintain protective immunity by rapidly expanding and differentiating into plasmablasts upon antigen exposure (213). Furthermore, a recent study showed that patients with cholera develop IgG, IgA, and IgM antibodies against NanH, and that plasma responses targeting this antigen correlate with protection (214). The protective role of other antibacterial antibodies against cholera is currently unknown.

Thus, this body of studies demonstrates that cholera infection stimulates innate cells at the site of infection, primarily neutrophils and their corresponding effector molecules. The subsequent adaptive response is characterized by Th1, Th17, and Tfh $CD4^+$ cells, which shape the antibody production targeting the CTX and various surface-exposed antigens. However, there are currently many knowledge gaps in understanding how these immunological processes occur. In this regard, to develop a highly effective cholera vaccine, it may be necessary to mimic these immune responses. As such, progress should be made in understanding the differences between the immune response triggered by *V. cholerae* infection and that induced by vaccination. This could pave the way for the development of the next generation of cholera vaccines.

7. Current vaccines licensed worldwide or with restricted license

The knowledge gained on immunity against *V. cholerae* has not only facilitated the development of current cholera vaccines, but also highlighted the possibility of developing novel vaccines that provide broader and longer-lasting protection. In this section, we will briefly review licensed cholera vaccines, while subsequent sections will focus on candidate vaccines currently undergoing clinical and preclinical evaluation.

In the 1960s, several large field studies conducted in Asian countries showed that injectable killed whole-cell cholera vaccines had modest efficacy and a high rate of adverse reactions, such as fever, local pain and swelling (215). Subsequently, interest shifted to exploring the potential of oral vaccination, which led to the development of the OCVs. Oral vaccines mainly interact with the immune system through the Waldeyer's tonsillar ring in the oral cavity or via the PP in the small intestine. Compared to vaccines administered by parenteral routes, oral vaccines have been found to induce stronger immune responses at the intestinal mucosa level, partly via antigen-specific sIgA (216). However, oral vaccines face some challenges, including the potential degradation of acid-labile antigens in the stomach, the lack of licensed oral adjuvants for human use, and the difficulty of their release at mucosal immune inductive sites (217).

At present, four OCVs based on killed whole cell vibrios are prequalified by the WHO (meaning that they can be purchased by United Nations agencies): Dukoral®, Shanchol™, Euvichol, and Euvichol-Plus (Table 3 and Box 2) (217).

Dukoral® was licensed in 1991 and since then has been distributed in over 60 countries. It is a monovalent vaccine composed of a mixture of three heat- or formalin-inactivated *V. cholerae* O1 strains (Classical Inaba strain Cairo 48, Classical Ogawa strain Cairo 50, and El Tor Inaba strain Phil 6973) along with the recombinant CTX-B (rCTX-B) subunit. The vaccine is free of the CTX-A subunit due to its toxicity. A sodium bicarbonate buffer is also added to the formulation to prevent the degradation of rCTX-B by gastric acid. This vaccine can be administered to individuals over 2 years of age and requires at least two doses for optimal efficacy. The protective efficacy (PE) of this vaccine has been demonstrated in several field trials in different countries, achieving over 80% protection in the first 6 months and gradually decreasing thereafter, ultimately providing negligible protection after 2 years. No significant severe adverse effects were attributed to this vaccine (220–222). Further analyses of volunteers vaccinated with Dukoral® revealed that this formulation induces high levels of specific sIgA antibodies and IFN-γ production in the duodenal mucosa (223). Notably, Dukoral® also provides significant protection for 3–9 months (PE: 67%) against diarrhea caused by enterotoxigenic *E. coli* (ETEC) producing heat-labile toxin (LT). This cross-protection is due to the cross-reactivity between the CTX-B subunit and LT (224).

In the late 1980s, the technology for manufacturing Dukoral® was transferred from Sweden to Vietnam for local production. This

BOX 2 Advantages and limitations of killed OCVs.

Killed OCVs possess several characteristics that make them effective in combating cholera:

- **Safety**: These vaccines have been proven safe, with only minor side effects reported.
- **Easy administration**: They can be easily administered in mass vaccination campaigns and in settings where injection-based vaccines may be logistically difficult to implement.
- **Cost-effectiveness**: Killed OCVs are relatively inexpensive, making them accessible to populations in resource-limited areas where cholera is prevalent.
- **Herd immunity**: OCVs not only protect the individuals who receive them but also create herd immunity, which can help to decrease transmission of the disease in the community (218, 219).

While killed OCVs offer several benefits as a tool for controlling cholera, they also have some limitations:

- **Limited effectiveness**: Their effectiveness can vary depending on the vaccinated population and the level of cholera transmission in the area. The protection provided by OCVs is short-term and decreases over time.
- **Limited immune response**: These vaccines do not contain live bacteria; thus, the immune response elicited may differ from that triggered by a natural cholera infection. This difference may result in a different pattern of immune response and antibody production, which can affect the duration and quality of the immunity provided.
- **Cold chain requirements**: They must be stored at a specific temperature range (typically between 2 and 8°C) to preserve their immunogenic properties, which can be challenging in areas with limited infrastructure.
- **Requirement for multiple doses**: They require at least two doses to provide adequate protection, which can be a barrier to achieving high coverage in some settings.
- **Limited role in outbreaks**: They do not provide immediate protection against cholera and are not intended to replace other control measures.

vaccine contained the same Dukoral strain composition, but the rCTX-B subunit was removed to simplify manufacturing, reduce costs, and improve stability. In 1992, the O139 epidemic in India and Bangladesh led to the addition of a killed O139 strain. This formulation was initially licensed in Vietnam as ORC-Vax™ and later after its modification as mORC-Vax™. It should be noted that the incorporation of the O139 component did not affect the responses to the original Dukoral components; instead, anti-O1 and anti-O139 antibodies were induced in serum and the intestinal mucosa (225, 226). However, the National Regulatory Agency (NRA) of Vietnam at that time did not have WHO approval, which limited the international use and WHO prequalification of this Vietnamese OCV. To make the vaccine available for international use, the manufacturing technology was transferred from Vietnam to Shantha Biotechnics in India, which had a WHO-approved NRA (195, 227). The PE of this vaccine was evaluated in a trial conducted in Kolkata, demonstrating that a two-dose immunization schedule provides an overall 65% protection over a five-year observation period. In 2009, this vaccine was licensed in India as Shanchol™, and WHO prequalified it in 2011 (228, 229).

The manufacturing technology of Shanchol™ was later transferred to Eubiologics in Seoul, Republic of Korea, resulting in the production of Euvichol®, which has an identical composition to Shanchol™. Studies in different countries have shown that Euvichol® and Shanchol™ elicit similar vibriocidal antibody responses and have comparable safety profiles. Euvichol® received licensure and WHO prequalification in 2015. Euvichol-Plus® is an improved vaccine that utilizes plastic tubes instead of conventional glass vials, providing better conditions for storage, transportation, and administration. This change has facilitated the delivery of this vaccine in emergency situations or humanitarian campaigns. Euvichol-Plus® received WHO prequalification in 2017 (230).

Two killed OCVs are licensed in some countries but are not WHO-prequalified. OraVacs™ is a dry formulation enteric-coated capsule vaccine containing a composition similar to Dukoral®. It is licensed in China and the Philippines (231). Cholvax™ is licensed in Bangladesh for use in the national cholera control program and has demonstrated safety and immunogenicity comparable to Shanchol™ (232).

The OCVs have achieved an important milestone in public health by providing herd immunity in vaccinated communities, thereby reducing person-to-person transmission (218, 219). In addition, the accumulation of evidence regarding the safety and efficacy of these vaccines has led the WHO to recommend their mass use as a preventive strategy in cholera-endemic areas, as well as a response measure during cholera outbreaks. Consequently, the WHO established the global OCV stockpile in 2011, which received support from the Global Alliance for Vaccines and Immunizations (Gavi Alliance) in 2014 (233). The main objectives of the OCV stockpile are to store and provide cholera vaccines during outbreaks and humanitarian campaigns, among other measures to control this disease. Presently, the OCV stockpile primarily uses Euvichol-Plus as its main formulation.

Despite their importance and usefulness, killed OCVs have several limitations. First, the PE of these vaccines is low (~42%) in children under the age of five, who are most vulnerable to the long-term effects and higher mortality associated with cholera (228). Second, they require multiple doses to achieve a high level of protection, which increases economic costs and the time required to elicit immunity. In fact, with a single dose, PE is only 8% for those under the age of five and 57.5% for those over the age of five (234, 235). Third, PE is short-term since it begins to decrease after 6 months and practically disappears after 3 or 5 years. In some sense, these limitations may be intrinsically related to the nature of killed vaccines. For instance, the *in vitro* cultures used to grow the vibrios included in these formulations do not reproduce host conditions and some important antigens may not be expressed. This is the case of the TcpA antigen, which is absent in the killed OCVs (236). Moreover, the formalin and heat treatment used to kill the bacteria may destroy or alter epitopes (237). Ultimately, killed vaccines are unable to mimic natural infection, so immune stimulation may be different from what is needed to achieve long-lasting protection.

Live attenuated OCVs have the potential to overcome many of the intrinsic limitations of killed OCVs. For instance, live attenuated vibrios closely mimic natural infection, and thus, they may trigger immune responses in the GALT, with the potential to target antigens

10.3389/fmed.2023.1155751

TABLE 3 Licensed cholera vaccines.

Vaccine	Manufacturer	Status	Composition	Immunization schedule	Duration of protection
Dukoral®	SBL vaccin, Sweden.	Licensed in 1991 in Sweden, and in more than 60 countries since then. WHO prequalification in 2001.	Monovalent vaccine containing heat- or formalin- killed strains of *V. cholerae* O1 (Classical Inaba strain Cairo 48, Classical Ogawa strain Cairo 50, and El Tor Inaba strain Phil 6,973), along with 1 mg rCTB.	Oral administration. For individuals aged 2 years and above. Children aged 2–5 years require 3 doses given 7–14 days apart, with a booster recommended after 6 months. Individuals aged 5 years and older require 2 doses given 7–14 days apart, with a booster recommended after 2 years.	Offers protection for 6 months to 2 years.
mORC-Vax™	VaBiotech, Vietnam.	Licensed in 1997 in Vietnam for local use only. Not WHO prequalified.	Bivalent vaccine containing heat- or formalin- killed strains of *V. cholerae* O1 (Classical Inaba strain Cairo 48, Classical Ogawa strain Cairo 50, and El Tor Inaba strain Phil 6,973) and the formalin-killed *V. cholerae* O139 strain 4260B.	Oral administration. For individuals aged 2 years and above. Two doses given 14 days apart. There is no booster recommendation from the manufacturer.	Offers protection for at least 3 years.
Shanchol™	Sanofi-Shantha Biotechnics, India.	Licensed in 2009 in India, and in 28 countries since then. WHO prequalification: 2011	Same composition as mORC-Vax™.	Oral administration. For individuals aged 1 year and above. Two doses given 14 days apart. There is no booster recommendation from the manufacturer.	Offers protection for at least 3–5 years.
Euvichol® / Euvichol-Plus®	Eubiologics, Republic of Korea.	WHO prequalification for Euvichol in 2015 and Euvichol-Plus in 2017.	Same composition as in Shanchol™.	Oral administration. For individuals aged 1 year and above. Two doses given 14 days apart. There is no booster recommendation from the manufacturer.	Not available.
OraVacs™	Shanghai United cell Biotechnology, China.	Licensed in China and the Philippines. Not WHO prequalified.	Dry formulation enteric-coated capsule vaccine containing a composition similar to Dukoral®.	Oral administration. For individuals aged 2 years and above. Three capsules taken on days 0, 7, and 28.	Not available.
Cholvax™	Incepta, Bangladesh.	Licensed in 2020 in Bangladesh. Not WHO prequalified.	Same composition as in Shanchol™	Oral administration. For individuals aged 1 year and above. Two doses given 14 days apart. There is no booster recommendation from the manufacturer.	Not available.
CVD 103-HgR (Vaxchora™)	PaxVax Inc., US.	Licensed in 2016 in USA, and in 2020 in Europe. Not WHO prequalified.	Live, attenuated *V. cholerae* O1 Classical Inaba strain CVD 103-HgR, a derivative of 569B.	Oral administration. For individuals between 2 and 64 years of age. Single dose (4×10^8 to 2×10^9 CFU).	Offers protection for 6 months.

which are only expressed *in vivo* during infection (199). Moreover, live attenuated OCVs may require a single dose, reducing the time required to achieve significant PE; this is particularly advantageous for individuals requiring travel at short notice to areas where an outbreak is occurring or where cholera is endemic (238).

Currently, only one live attenuated OCV is available, named Vaxchora™, which is approved in the United States and Europe for travelers visiting regions where cholera is endemic (238). The approval of Vaxchora™ in other markets is pending. This vaccine is based on the *V. cholerae* strain CVD 103-HgR, serogroup O1, serotype Inaba,

classical biotype, which is derived from the strain 569B. The CVD 103-HgR strain is genetically modified and contains a deletion of the *ctxA* gene and an insertion of the Hg++ resistance gene to enable differentiation of the vaccine strain from the wild type (239). Although the CVD 103-HgR strain cannot produce active CTX, it can synthesize the CTX-B subunit and the TcpA antigen, and colonize the small intestine transiently (199). The initial CVD 103-HgR formulation was introduced in 1993, and since then, it has been manufactured by various companies and known by other trade names such as Orochol®, Mutacol®, and Orochol-E®.

The effectiveness of the CVD 103-HgR vaccine was initially evaluated in four experimental challenge studies between 1987 and 1999, where the PE against severe diarrhea was 92.7, 95.4, 79.0, and 67.6% (239). Moreover, this vaccine can elicit a significant VAT 10 days after immunization, but the duration of protection has not been fully determined (240, 241). However, conflicting results were obtained in two field studies conducted in North Jakarta, Indonesia, between 1993 and 1997 (242), and on the island of Pohnpei, Micronesia, during an outbreak in 2001 (243). The PE obtained in the Indonesian study was only 14%, whereas in the Micronesian study, it was 79.2%. The poor performance of this vaccine in the Indonesian study was attributed to a lower-than-expected cholera incidence (242). Thus, the effectiveness of Vaxchora™ in cholera-endemic areas remains unclear.

Additionally, several factors have limited the use of the CVD 103-HgR vaccine beyond the traveler's market, including possible toxigenic reversal, high cost, and the requirement of a cold chain (−25 to −15°C) (244). For further details beyond what is provided here on the CVD 103-HgR vaccine and the history of its development, the reader is referred to recent comprehensive reviews (238, 239, 244).

8. Vaccines candidates in clinical development

Much work has been done in recent years to improve the manufacturing process of killed OVCs, to enhance their stability, and to further reduce costs. An example of this is Hillchol® (245), which was developed by Bharat Biotech International in India. Hillchol® is based on the formalin-killed *V. cholerae* O1 Hikojima strain MS1568 (Table 4). The MS1568 strain is a derivative of Phil 6973 strain, which is a component of Shanchol™. It has a partially inactivating mutation in the *wbeT* gene that is responsible for LPS methylation, which differentiates the Ogawa and Inaba serotypes; thus, this strain expresses ~50% of both LPS. As a result, Hillchol® requires a single-strain manufacturing process that is less expensive than other killed OCVs but still maintains a mixed O1 antigen composition (36). Hillchol® completed a phase I/II study evaluating its safety, tolerability and immunogenicity. The study demonstrated that it is not inferior to Shanchol™ in individuals of different age groups residing in a cholera-endemic region (246). In August 2022, Hillchol® began a phase III study (Clinical Trial NCT 05507229).

Over the past three decades, several live attenuated OCV candidates have been developed. However, only four of them have progressed to clinical trials. The oldest among them, CholeraGarde® (Peru-15), was reported in 1995. It is based on a *V. cholerae* O1 El Tor Inaba strain derived from the C6709 strain, which was isolated in Peru in 1991. The Peru-15 strain is attenuated due to a deletion of the CTXΦ prophage and a spontaneous mutation that affects motility. It also has an insertion of the *ctxB* gene in the *recA* gene for the constitutive expression of the CTX-B subunit. Since the *recA* gene is required for homologous recombination, the Peru-15 strain has a reduced capacity for horizontal gene transfer (247, 248). CholeraGarde® was shown to be safe and immunogenic in phase I/II studies conducted in the United States, Bangladesh and Thailand (249–252). In challenge studies, a single dose of this formulation demonstrated a PE of 100% against moderate and severe diarrhea. Additionally, only a small percentage of individuals (7%) developed mild diarrhea after challenge (253). The last clinical trial of this vaccine candidate was reported in 2015, and it is unclear whether it will be evaluated in a phase III study.

Another live attenuated OCV candidate, Vax-COLER® (Cuban 638), was reported in 1999. It is based on the *V. cholerae* El Tor Ogawa strain 638, which is derived from the C7258 strain isolated in Peru in 1991. The 638 strain is attenuated due to the deletion of the CTXΦ prophage and an insertion of the *Clostridium thermocellum* endoglucanase A (*celA*) gene into the hemagglutinin/protease (*hapA*) gene (254). Vax-COLER® has been shown to be safe and immunogenic in phase I/II studies conducted in Cuba and in a cholera endemic area in Maputo, Mozambique (254–256). It has also been found to provide protection against a challenge with the *V. cholerae* O1 El Tor strain 3,008 (257). However, there is currently no available information on whether this vaccine candidate will be evaluated in a phase III study.

A third live attenuated OCV candidate is VA1.4, which was initially reported in 1999 as VA1.3 (258). The VA1.3 is a non-toxigenic *V. cholerae* O1 El Tor Inaba strain with an insertion of the *ctxB* gene (under the control of the *ctx* promoter) into the *hlyA* gene. This strain naturally lacked the CTXΦ prophage and has proven to be non-reactogenic in a rabbit ileal loop assay. In 2009, a phase I/II study conducted in a cholera-endemic area in Kolkata, India, showed that the VA1.3 strain is safe and immunogenic (259). A later version of this vaccine candidate is the VA1.4 strain, which is identical to VA1.3, except for the absence of an ampicillin resistance gene. In 2014, a phase I/II study conducted in Kolkata, India, showed that VA1.4 is also safe and immunogenic (260). This vaccine candidate was evaluated with a two-dose schedule of 1.9×10^9 CFU, but no additional benefit was observed after the second dose. Currently, there is no information available regarding a phase III study for this formulation.

The fourth and most recent live attenuated OCV candidate is PanChol (HaitiV), which was developed in 2018 in the USA (261). HaitiV is derived from a variant O1 El Tor Ogawa strain isolated during the 2010 Haiti outbreak. The HaitiV strain has several genetic modifications that make it avirulent and resistant to reversion, but it maintains the ability to colonize the intestine and induce immune responses. These genetic modifications include deletions of: (i) the entire CTXΦ and its boundaries encoding the MARTX toxin (*rtxABCDE*) genes; (ii) the *hupB* gene required for episomal maintenance of CTXΦ; (iii) five flagellin subunits (*flaA-E*) genes; (iv) a region of DNA containing resistance genes for the antibiotics trimethoprim (*dfrA*), sulfamethoxazole (*sul2*), streptomycin (*strAB*), and chloramphenicol (*floR*); and (v) the *recA* gene involved in gene acquisition by homologous recombination. In addition, HaitiV has an insertion of the *ctxB* gene (under the control of the *htpG* promoter) in the neutral locus N900_11550. To prevent toxigenic reversion, the HaitiV strain also encodes a CRISPR/Cas9 system targeting the *ctxA* gene.

TABLE 4 Cholera vaccine candidates under clinical evaluation.

Vaccine	Manufacturer	Status	Composition	Immunization schedule	Duration of protection
Hillchol®	Bharat Biotech International Ltd., India.	Completed phase I/phase II clinical safety and immunogenicity study. Phase III clinical study underway (Clinical Trial NCT 05507229).	Monovalent vaccine containing formalin-killed whole cell of recombinant *V. cholerae* O1 El Tor Hikojima strain MS1568, which expresses ~50% each of Ogawa and Inaba LPS.	Oral administration. For individuals between 1 and 45 years of age. Two doses (under study).	Not available.
CholeraGarde® (Peru-15)	Vaccine Technologies Inc., USA.	Completed phase I/phase II clinical safety and immunogenicity studies, last reported in 2015.	Live, attenuated, non-motile, *V. cholerae* O1 El Tor Inaba strain C6709 (ΔCTXΦ *ctxB*::rec*A*).	Oral administration. Single dose (Up to 1×10^9 CFU) for healthy adults, children above aged 9 months, and in HIV-positive adults (aged 18–45 years).	Not available.
Vax-COLER® (Cuban 638)	Finlay Institute, Havana, Cuba.	Completed phase I/phase II clinical safety and immunogenicity study, last reported in 2011.	Live, attenuated *V. cholerae* O1 El Tor Ogawa 638 (ΔCTXΦ *hapA*::*celA*).	Oral administration. Single dose (2×10^9 CFU) for individuals between 18 and 50 years of age.	Not available.
VA 1.3 / VA 1.4	Shantha Biotech, India.	Completed phase I/phase II clinical safety and immunogenicity study, last reported in 2014.	Live, attenuated, non-toxigenic *V. cholerae* O1 El Tor Inaba (Δ*hlyA*::*ctxB*).	Oral administration. Single and double dose (1.9×10^9 CFU) for individuals between 18 and 60 years of age.	Not available.
Panchol (HaitiV)	Harvard University, USA.	Phase 1 clinical study underway (Clinical Trial NCT 05657782).	Live, attenuated *V. cholerae* O1 El Tor Ogawa strain HaitiV, with nine genetically engineered mutations.	Oral administration. Single dose. CFU concentrations under study: log10 values 6, 7, 8, 9 and 10.	Not available.
OSP:rTTHc	Eubiologics Ltd., South Korea, and Harvard University, USA.	Phase 1 clinical study underway (Clinical Trial NCT 05559983).	Conjugated vaccine candidate containing Inaba or Ogawa OSP linked to recombinant tetanus toxoid heavy chain fragment (rTThc), with or without aluminum phosphate adjuvant.	Immunization schedule under study: two doses of 5, 10, and 25 μg, with or without aluminum phosphate adjuvant, administered intramuscularly 4 weeks apart.	Not available.
MucoRice-CTB (IMSUT-MR1501)	University of Tokyo, Japan.	Completed phase 1 clinical study (UMIN Clinical Trials Registry UMIN000018001)	Oral rice-based vaccine expressing CTX-B subunit.	Oral administration. 6 g once every 2 weeks for 8 weeks (for a total of 4 doses).	Not available.

It should be noted that oral administration of HaitiV in animal models has demonstrated a protective effect within 24 h post-vaccination against a lethal dose of the parent *V. cholerae* strain HaitiWT (261, 262). This rapid protection was achieved before the induction of any adaptive immune response, suggesting that HaitiV exhibits a probiotic-like activity. However, it is unclear whether this "probiotic" effect is specific to HaitiV or also present in other live attenuated OVCs. Moreover, immunization of mice with this vaccine candidate was well-tolerated and immunogenic, triggering humoral responses consisting of anti-OSP and anti-CTX-B IgM, IgG, and IgA antibodies. In December 2022, PanChol began a phase I study for safety, tolerability, and immunogenicity in healthy volunteers (Clinical Trial NCT05657782).

As previously mentioned, protection against cholera is mainly serogroup-specific. Furthermore, the generation of anti-OSP antibodies is a common immune response elicited by various cholera vaccines, and these antibodies have been associated with protection in both animal models and in humans. This has been the rationale for the use of LPS and the O-antigen as a target for the development of cholera vaccines. In this regard, vaccine candidates based on the O-antigen conjugated with protein carriers are an interesting alternative to OCVs.

One of the first cholera conjugate vaccine candidates was prepared by binding the detoxified (deacylated) LPS (DeA-LPS) with the CTX (263). Subsequent evaluation of the DeA-LPS-CTX conjugate in a phase I study in adult volunteers showed that it was immunogenic by eliciting vibriocidal (anti-LPS) antibodies and IgG anti-CTX antibodies (264). However, this vaccine candidate was not further evaluated.

More recently, cholera conjugate vaccine candidates were developed by binding the Inaba or Ogawa OSP with the recombinant tetanus toxoid heavy chain fragment (rTThc). Preclinical evaluation of the OSP: rTTHc conjugates has shown that they are immunogenic and protective in mice (265, 266). Interestingly, a combined vaccination approach which includes an oral priming with Vaxchora™ followed by a parenteral boost with the OSP: rTTHc conjugate resulted in increased immune responses in mice (267). In 2021, the OSP: rTTHc conjugate candidate was produced in a scalable manner, and the addition of aluminum phosphate adjuvant increased the OSP-specific immune responses in mice (268). In September 2022, the OSP: rTTHc conjugate vaccine began a phase I study primarily to determine the safety of the dose range with or without aluminum phosphate adjuvant, and secondarily to assess humoral immune responses in the nonendemic population, which will guide the selection of future doses (Clinical Trial NCT 05559983).

Plant-based vaccines represent a step toward new vaccinology technologies and oral vaccination. These innovative vaccines have some advantages over classical vaccines, including the long-term preservation of antigenic proteins without the need for a cold chain, resistance to digestion in the stomach, lower cost, increased safety, and scalability (269). Potato, tomato, and rice are attractive antigen-expressing plants that have been used as a platform for the development of candidate vaccines against some infectious diseases in animals and humans (269–271). Notably, the expression of CTX-B subunit oligomers has been reported in transgenic potato (272), tomato (273, 274), and rice plants (275, 276). Additionally, the TcpA antigen has also been expressed in transgenic tomato plants (277). However, transgenic rice expressing the CTX-B subunit has been by far the most studied.

In 2007, MucoRice-CTB, a transgenic rice-based vaccine expressing the CTX-B subunit, was developed. This platform produced an average of 30 μg of recombinant CTX-B per transgenic rice seed, which was stored in protein bodies (PBs), a type of storage organelle in rice. The *in vitro* assays with pepsin showed that the CTX-B was not degraded, suggesting that the PBs may act as a natural capsule for oral administration of the vaccine. Preclinical studies in mice and pigs orally immunized with the seed powder showed that MucoRice-CTB induced CTX-B-specific serum IgG and intestinal sIgA antibodies (275, 278–281). In the intestinal loop assay, the sIgA antibodies that were generated were found to confer protection against *V. cholerae* and LT-ETEC challenges (278). However, this formulation was not evaluated in an animal challenge assay to test whether it conferred protection against colonization by *V. cholerae*. This is probably because CTX-B-specific antibodies do not have vibriocidal activity. As a step toward the use of MucoRice-CTB in humans, this vaccine candidate was evaluated in non-human primates (*Macaca fascicularis*), inducing CTX-B-specific antibodies without adverse effects (279). Recently, a phase I study conducted in Japan showed that MucoRice-CTB increased CTB-specific serum IgG and IgA antibody levels without inducing serious adverse events. A similar phase 1 study is planned with individuals of other ethnicities (282).

9. Vaccine candidates in preclinical development

Some live OCVs were developed and evaluated in animal models several years ago, including IEM108 (283, 284), TLP01 (285), and VCUSM2 (286). However, no further related studies have been published since then. Although mentioned for historical reasons, interested readers are recommended to refer to earlier reviews where these vaccine candidates have already been discussed (287, 288).

Recent technological advances in vaccine design and manufacture have led to promising cholerae vaccine candidates, such as DuoChol™. This killed OCV is a lyophilized mixture of formalin-killed isogenic El Tor Ogawa and Inaba strains and rCTB in an enterocoated capsule. This formulation improves thermostability and could facilitate its integration into standard immunization programs in cholera-endemic areas. DuoChol™ is currently in preclinical development at the University of Gothenburg, Sweden (215, 227).

OMVs have emerged as a promising strategy for developing vaccines against Gram-negative bacterial pathogens, including *V. cholerae*. *V. cholerae* OMVs contain important virulence factors such as CTX, TcpA, OmpU, NanH, LPS, and others (70–72, 289, 290). Several preparations of OMVs derived from WT or mutant *V. cholerae* strains have been administered to mice through different routes, resulting in strong humoral responses against a variety of OMV-associated antigens. Immunization with OMVs protects against *V. cholerae* colonization regardless of the route of administration (291). In particular, intranasal immunization with OMVs induces O-specific antibodies, particularly IgG, which inhibit *V. cholerae* motility (292, 293). In another study, Leitner et al. (290) developed a combined formulation of OMVs derived from *V. cholerae* and ETEC. Interestingly, this OMV mixture conferred protection in mice against both pathogens, suggesting the potential for developing a broadly protective OMV-based vaccine against several Gram-negative pathogens by combining OMVs.

Virus-like particles (VLPs) are multi-protein structures that mimic the organization and conformation of native viruses, but lack the viral genome, making them a safe template for vaccine development (294). Over the past three decades, VLPs have served as a successful platform for developing vaccines against various viral diseases (295). However, their potential use against non-viral pathogens has scarcely been explored. A recent study reported the coupling of VLPs from the bacteriophage Qβ to the *V. cholerae* OSP antigen, which was immunogenic in mice, eliciting IgG antibodies with vibriocidal activity (296).

The development of chimeric proteins is a growing trend in the design of next-generation vaccines. The biotechnological revolution, particularly the improvements in gene synthesis, has opened new doors for the rational design of protein-based vaccines (297). Chimeric proteins carrying selected epitopes from several strains or different pathogens can enhance the immunogenicity of the recombinant antigen, eliciting a broader immune response (298). Chimeric protein-based vaccines against cholera have focused on

known antigenic proteins, including the CTX-A and CTX-B subunits, flagellins, OmpW, OmpU, TcpA, TcpF, and NanH. Two vaccine candidates, TcpF-CTA2-CT-B and TcpA-CTA2-CT-B, are chimeric proteins (299, 300). Both chimeras were immunogenic in mice and triggered specific antibodies that conferred protection in passively immunized infant mice. However, no further studies have been published regarding these vaccine candidates. Similar results were obtained by the OTC chimera (OmpW, TcpA, and CTX-B), which elicited specific IgG antibodies that were protective in the ileal loop assay and in passively immunized infant mice (301).

An interesting recent study describes the polyvalent cholera MEFA protein, which contains antigenic domains of TcpA, CTX, NanH, HlyA, flagellins, and peptides mimicking the OSP on a flagellin B backbone (302). Mice and rabbits immunized intramuscularly with the MEFA protein developed antibodies to all the virulence factors targeted by the immunogen, except LPS. The antibodies generated neutralized CTX, bacterial motility, and *in vitro* adherence of *V. cholerae* O1, O139, and non-O1/non-O139 strains. Moreover, this vaccine provided cross-protective against *V. cholerae* O1, O139, and non-O1/non-O139 strains in adult and infant rabbit colonization models.

Despite promising results, protein-based vaccines have several limitations. For instance, they are often poorly immunogenic and require multiple doses and adjuvants to achieve protective immunity. In addition, they are generally administered parenterally to avoid enzymatic degradation in the stomach, inducing strong humoral responses at the systemic level but not at the intestinal mucosa level. Although this type of vaccine represents a potential alternative to OCVs, none of them have been tested in human trials. More importantly, they must compete in a market that demands cholera vaccines that are cost-effective and administered in a single-dose regimen.

10. Concluding remarks and prospects

Over the last few decades, much knowledge has been gained about the pathogenesis and immune response of *V. cholerae* infection, which has resulted in the development of treatments and vaccines. However, progress toward a highly effective cholera vaccine has been hindered by several limitations. These include the lack of a well-defined correlate of long-term protective immunity as well as an animal model that fully recapitulates the disease (303). In addition, it is largely unknown how the microbiota confers resistance or susceptibility to cholera and how it affects the immune response generated by vaccines against this disease (125). In this respect, human microbiota-associated mice could be a valuable animal model to consider (30).

Further studies are needed to investigate how immune responses are produced during *V. cholerae* infection. In particular, it is important to understand the innate immune pathways that are modulated during the natural course of infection, and whether these responses are beneficial or detrimental to the host. Additionally, it is crucial to clarify how long-term immune memory is generated in patients recovering from cholera. This information is essential because a highly effective cholera vaccine must recapitulate or mimic these immune responses.

OCVs have been shown to be safe, and although they confer short-term protection, their usefulness in cholera control has been reliably demonstrated. It is likely that new oral adjuvants, such as nanocarriers (304), lipid-based adjuvants (305), among other (306–308), could increase the efficacy of these vaccines.

The OMV-based vaccines, plant-based vaccines, and chimeric antigens are emerging and promising approaches in vaccine development. Moreover, mRNA vaccines against SARS-CoV-2 have been rapidly developed and have proven to be highly efficacious and adaptable as required. Recent studies have demonstrated the potential of mRNA vaccines against bacterial pathogens (309–311). Therefore, new cholera vaccine candidates based on these platforms are expected to appear in the coming years.

Another strategy to improve cholera vaccines could be the development of multivalent vaccines that protect against various enteric pathogens. Finally, in the human-pathogen arms race, the development of new vaccine technologies is likely the key factor in winning the battle and, ideally, in finding a highly long-lasting protective cholera vaccine.

Author contributions

DM and RV wrote the draft of the manuscript. MO'R edited the manuscript. All authors contributed to data acquisition and analysis and approved the publication of the content.

Funding

This work was supported by Postdoctoral FONDECYT grant 3190524, awarded to DM. Regular FONDECYT grant 1211647, awarded to RV.

Acknowledgments

In memory of Carmenza Forero (1955 – 2020), beloved mother and best teacher. We thank Helen Lowry for the careful revision and edition of the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Sit B, Fakoya B, Waldor MK. Emerging concepts in cholera vaccine design. *Annu Rev Microbiol*. (2022) 76:681–702. doi: 10.1146/annurev-micro-041320-033201

2. Williams PCM, Berkley JA. Guidelines for the management of paediatric cholera infection: a systematic review of the evidence. *Paediatr Int Child Health*. (2018) 38:S16–31. doi: 10.1080/20469047.2017.1409452

3. Ganesan D, Sen GS, Legros D. Cholera surveillance and estimation of burden of cholera. *Vaccine*. (2020) 38:A13–7. doi: 10.1016/j.vaccine.2019.07.036

4. Domman D, Chowdhury F, Khan AI, Dorman MJ, Mutreja A, Uddin MI, et al. Defining endemic cholera at three levels of spatiotemporal resolution within Bangladesh. *Nat Genet*. (2018) 50:951–5. doi: 10.1038/s41588-018-0150-8

5. Ingelbeen B, Hendrickx D, Miwanda B, van der Sande MAB, Mossoko M, Vochten H, et al. Recurrent cholera outbreaks, democratic republic of the Congo, 2008–2017. *Emerg Infect Dis*. (2019) 25:856–64. doi: 10.3201/eid2505.181141

6. Zheng Q, Luquero FJ, Ciglenecki I, Wamala JF, Abubakar A, Welo P, et al. Cholera outbreaks in sub-Saharan Africa during 2010-2019: a descriptive analysis. *Int J Infect Dis*. (2022) 122:215–21. doi: 10.1016/j.ijid.2022.05.039

7. Muzembo BA, Kitahara K, Debnath A, Ohno A, Okamoto K, Miyoshi SI. Cholera outbreaks in India, 2011–2020: a systematic review. *Int J Environ Res Public Health*. (2022) 19:2011–20. doi: 10.3390/ijerph19095738

8. Charnley GEC, Kelman I, Murray KA. Drought-related cholera outbreaks in Africa and the implications for climate change: a narrative review. *Pathog Glob Health*. (2022) 116:3–12. doi: 10.1080/20477724.2021.1981716

9. Ali M, Nelson AR, Lopez AL, Sack DA. Updated global burden of cholera in endemic countries. *PLoS Negl Trop Dis*. (2015) 9:e0003832. doi: 10.1371/journal.pntd.0003832

10. Legros D. Global cholera epidemiology: opportunities to reduce the burden of cholera by 2030. *J Infect Dis*. (2018) 218:S137–40. doi: 10.1093/infdis/jiy486

11. Thakur S, Chauhan V. State of the globe: revisiting cholera – the larger ongoing pandemic. *J Glob Infect Dis*. (2021) 13:113. doi: 10.4103/jgid.jgid_234_21

12. Musa SS, Gyeltshen D, Manirambona E, Wada YH, Sani AF, Ullah I, et al. Dual tension as Nigeria battles cholera during the COVID-19 pandemic. *Clin Epidemiol Glob Heal*. (2021) 12:100913. doi: 10.1016/j.cegh.2021.100913

13. World Health Organization. Cholera, 2020. *Wkly Epidemiol Rec*. (2020) 96:445–54. Available at: https://apps.who.int/iris/bitstream/handle/10665/345271/WER9637-445-454-eng-fre.pdf?sequence=1&isAllowed=y

14. World Health Organization. *Wkly Epidemiol Rec*. (2022) 97:Cholera, 2021:453–64. Available at: https://apps.who.int/iris/handle/10665/362858

15. Amani A, Fouda AAB, Nangmo AJ, Bama SN, Tatang CA, Mbang MA, et al. Reactive mass vaccination campaign against cholera in the covid-19 context in Cameroon: challenges, best practices and lessons learned. *Pan Afr Med J*. (2021) 38:392. doi: 10.11604/pamj.2021.38.392.27754

16. Taylor DL, Kahawita TM, Cairncross S, Ensink JHJ. The impact of water, sanitation and hygiene interventions to control cholera: a systematic review. *PLoS One*. (2015) 10:e0135676. doi: 10.1371/journal.pone.0135676

17. Ramamurthy T, Das B, Chakraborty S, Mukhopadhyay AK, Sack DA. Diagnostic techniques for rapid detection of *Vibrio cholerae* O1/O139. *Vaccine*. (2020) 38:A73–82. doi: 10.1016/j.vaccine.2019.07.099

18. Abramo JM, Reynolds A, Crisp GT, Weurlander M, Söderberg M, Scheja M, et al. Individuality in music performance. *Assess Eval High Educ*. (2012) 37:435. doi: 10.1007/82

19. Farmer P, Almazor CP, Bahnsen ET, Barry D, Bazile J, Bloom BR, et al. Meeting Cholera's challenge to Haiti and the world: a joint statement on cholera prevention and care. *PLoS Negl Trop Dis*. (2011) 5:e1145. doi: 10.1371/journal.pntd.0001145

20. Das M, Singh H, Girish Kumar CP, John D, Panda S, Mehendale SM. Non-vaccine strategies for cholera prevention and control: India's preparedness for the global roadmap. *Vaccine*. (2020) 38:A167–74. doi: 10.1016/j.vaccine.2019.08.010

21. Chatterjee P, Kanungo S, Bhattacharya SK, Dutta S. Mapping cholera outbreaks and antibiotic resistant *Vibrio cholerae* in India: an assessment of existing data and a scoping review of the literature. *Vaccine*. (2020) 38:A93–A104. doi: 10.1016/j.vaccine.2019.12.003

22. World Health Organization. Ending cholera: a global roadmap to 2030. (2017). Available at: https://www.gtfcc.org/wp-content/uploads/2019/10/gtfcc-ending-cholera-a-global-roadmap-to-2030.pdf (accessed July 12, 2022)

23. Michel E, Gaudart J, Beaulieu S, Bulit G, Piarroux M, Boncy J, et al. Estimating effectiveness of case-area targeted response interventions against cholera in Haiti. *Elife*. (2019) 8:1–34. doi: 10.7554/eLife.50243

24. Reveiz L, Chapman E, Ramon-Pardo P, Koehlmoos TP, Cuervo LG, Aldighieri S, et al. Chemoprophylaxis in contacts of patients with cholera: systematic review and meta-analysis. *PLoS One*. (2011) 6:6–11. doi: 10.1371/journal.pone.0027060

25. Gabutti G, Rossanese A, Tomasi A, Giuffrida S, Nicosia V, Barriga J, et al. Cholera the current status of cholera vaccines and recommendations for travellers. *Vaccine*. (2020) 8:1–17. doi: 10.3390/vaccines8040606

26. Bornside GH. Jaime Ferran and preventive inoculation against cholera. *Bull Hist Med*. (1981) 55:516–32.

27. Bornside GH. Waldemar Haffkine's cholera vaccines and the Ferran-Haffkine priority dispute. *J Hist Med Allied Sci*. (1982) 37:399–422. doi: 10.1093/jhmas/XXXVII.4.399

28. Sack DA, Sack RB, Nair GB, Siddique A. Cholera (the lancet). *Lancet*. (2004) 363:223–33. doi: 10.1016/S0140-6736(03)15328-7

29. Levine MM. Developing countries: lessons from a live cholera vaccine. *BMC Biol*. (2010) 8:129. doi: 10.1186/1741-7007-8-129

30. Macbeth JC, Liu R, Alavi S, Hsiao A. A dysbiotic gut microbiome suppresses antibody mediated-protection against *Vibrio cholerae*. *iScience*. (2021) 24:103443. doi: 10.1016/j.isci.2021.103443

31. Dutta D, Chowdhury G, Pazhani GP, Guin S, Dutta S, Ghosh S, et al. *Vibrio cholerae* non-O1, non-O139 serogroups and cholera-like diarrhea, Kolkata. *India Emerg Infect Dis*. (2013) 19:464–7. doi: 10.3201/eid1903.121156

32. Arteaga M, Velasco J, Rodriguez S, Vidal M, Arellano C, Silva F, et al. Genomic characterization of the non-O1/non-O139 *Vibrio cholerae* strain that caused a gastroenteritis outbreak in Santiago, Chile, 2018. *Microb Genomics*. (2020) 6:e000340. doi: 10.1099/mgen.0.000340

33. Octavia S, Salim A, Kurniawan J, Lam C, Leung Q, Ahsan S, et al. Population structure and evolution of non-O1/non-O139 *Vibrio cholerae* by multilocus sequence typing. *PLoS One*. (2013) 8:e65342. doi: 10.1371/journal.pone.0065342

34. Chen Y, Bystricky P, Adeyeye J, Panigrahi P, Ali A, Johnson JA, et al. The capsule polysaccharide structure and biogenesis for non-O1 *Vibrio cholerae* NRT36S: genes are embedded in the LPS region. *BMC Microbiol*. (2007) 7:1–15. doi: 10.1186/1471-2180-7-20

35. Stroeher UH, Karageorgos LE, Morona R, Manning PA. Serotype conversion in *Vibrio cholerae* O1. *Proc Natl Acad Sci U S A*. (1992) 89:2566–70. doi: 10.1073/pnas.89.7.2566

36. Karlsson SL, Ax E, Nygren E, Källgård S, Blomquist M, Ekman A, et al. Development of stable *Vibrio cholerae* O1 Hikojima type vaccine strains co-expressing the Inaba and Ogawa lipopolysaccharide antigens. *PLoS One*. (2014) 9:e108521. doi: 10.1371/journal.pone.0108521

37. Mohammadi Barzelighi H, Bakhshi B, Boustanshenas M. Genetic determinants differences between *Vibrio cholerae* biotypes. *Infect Epidemiol Med*. (2016) 2:26–30. doi: 10.18869/modares.iem.2.2.26

38. Clemens JD, Nair GB, Ahmed T, Qadri F, Holmgren J. Cholera. *Lancet*. (2017) 390:1539–49. doi: 10.1016/S0140-6736(17)30559-7

39. Nair GB, Qadri F, Holmgren J, Svennerholm AM, Safa A, Bhuiyan NA, et al. Cholera due to altered El Tor strains of *Vibrio cholerae* O1 in Bangladesh. *J Clin Microbiol*. (2006) 44:4211–3. doi: 10.1128/JCM.01304-06

40. Devault AM, Golding GB, Waglechner N, Enk JM, Kuch M, Tien JH, et al. Second-pandemic strain of *Vibrio cholerae* from the Philadelphia cholera outbreak of 1849. *N Engl J Med*. (2014) 370:334–40. doi: 10.1056/NEJMoa1308663

41. Chowdhury F, Ross AG, Islam MT, McMillan NAJ, Qadri F. Diagnosis, management, and future control of cholera. *Clin Microbiol Rev*. (2022) 35:e0021121. doi: 10.1128/cmr.00211-21

42. Samadi AR, Shahid N, Eusof A, Yunus M, Huq MI, Khan MU, et al. Classical *Vibrio cholerae* biotype displaces EL tor in Bangladesh. *Lancet*. (1983) 321:805–7. doi: 10.1016/S0140-6736(83)91860-3

43. Mutreja A, Kim DW, Thomson NR, Connor TR, Lee JH, Kariuki S, et al. Evidence for several waves of global transmission in the seventh cholera pandemic. *Nature*. (2011) 477:462–5. doi: 10.1038/nature10392

44. Weill F, Domman D, Njamkepo E, Tarr C, Rauzier J, Fawal N, et al. Genomic history of the seventh pandemic of cholera in Africa. *Science*. (2017) 358:785–9. doi: 10.1126/science.aad5901

45. Bhandari M, Jennison AV, Rathnayake IU, Huygens F. Evolution, distribution and genetics of atypical *Vibrio cholerae* – a review. *Infect Genet Evol*. (2021) 89:104726. doi: 10.1016/j.meegid.2021.104726

46. Mukhopadhyay AK, Takeda Y, Balakrish NG. Cholera outbreaks in the El Tor biotype era and the impact of the new El Tor variants. *Assess Eval High Educ*. (2014) 379:17–47. doi: 10.1007/82_2014_363

47. Kim EJ, Lee CH, Nair GB, Kim DW. Whole-genome sequence comparisons reveal the evolution of *Vibrio cholerae* O1. *Trends Microbiol*. (2015) 23:479–89. doi: 10.1016/j.tim.2015.03.010

48. Okada K, Iida T, Kita-Tsukamoto K, Honda T. Vibrios commonly possess two chromosomes. *J Bacteriol*. (2005) 187:752–7. doi: 10.1128/JB.187.2.752-757.2005

49. Heidelberg JF, Elsen JA, Nelson WC, Clayton RA, Gwinn ML, Dodson RJ, et al. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. *Nature*. (2000) 406:477–83. doi: 10.1038/35020000

50. Kimsey HH, Waldor MK. CTXφ immunity: application in the development of cholera vaccines. *Proc Natl Acad Sci U S A*. (1998) 95:7035–9. doi: 10.1073/pnas.95.12.7035

51. Hassan F, Kamruzzaman M, Mekalanos JJ, Faruque SM. Satellite phage TLC φ enables toxigenic conversion by CTX phage through dif site alteration. *Nature*. (2010) 467:982–5. doi: 10.1038/nature09469

52. Rajanna C, Wang J, Zhang D, Xu Z, Ali A, Hou YM, et al. The vibrio pathogenicity Island of epidemic *Vibrio cholerae* forms precise extrachromosomal circular excision products. *J Bacteriol*. (2003) 185:6893–901. doi: 10.1128/JB.185.23.6893-6901.2003

53. Jermyn WS, Boyd EF. Characterization of a novel vibrio pathogenicity island (VPI-2) encoding neuraminidase (nanH) among toxigenic *Vibrio cholerae* isolates. *Microbiology*. (2002) 148:3681–93. doi: 10.1099/00221287-148-11-3681

54. Davies BW, Bogard RW, Young TS, Mekalanos JJ. Coordinated regulation of accessory genetic elements produces cyclic di-nucleotides for *V. cholerae* virulence. *Cells*. (2012) 149:358–70. doi: 10.1016/j.cell.2012.01.053

55. O'Shea YA, Finnan S, Reen FJ, Morrissey JP, O'Gara F, Boyd EF. The Vibrio seventh pandemic island-II is a 26·9 kb genomic island present in *Vibrio cholerae* El Tor and O139 serogroup isolates that shows homology to a 43·4 kb genomic island in *V. vulnificus*. *Microbiology*. (2004) 150:4053–63. doi: 10.1099/mic.0.27172-0

56. Waldor MK, Tschäpe H, Mekalanos JJ. A new type of conjugative transposon encodes resistance to sulfamethoxazole, trimethoprim, and streptomycin in *Vibrio cholerae* O139. *J Bacteriol*. (1996) 178:4157–65. doi: 10.1128/jb.178.14.4157-4165.1996

57. Mazel D, Dychinco B, Webb VA, Davies J. A distinctive class of integron in the *Vibrio cholerae* genome. *Science*. (1998) 280:605–8. doi: 10.1126/science.280.5363.605

58. Murphy RA, Fidelma BE. Three Pathogenicity Islands of *Vibrio cholerae* can excise from the chromosome and form circular intermediates †. *J Bacteriol*. (2008) 190:636–47. doi: 10.1128/JB.00562-07

59. Pant A, Bag S, Saha B, Verma J, Kumar P, Banerjee S, et al. Molecular insights into the genome dynamics and interactions between core and acquired genomes of *Vibrio cholerae*. *Proc Natl Acad Sci U S A*. (2020) 117:23762–73. doi: 10.1073/pnas.2006283117

60. Faruque SM, Chowdhury N, Kamruzzaman M, Dziejman M, Rahman MH, Sack DA, et al. Genetic diversity and virulence potential of environmental *Vibrio cholerae* population in a cholera-endemic area. *Proc Natl Acad Sci U S A*. (2004) 101:2123–8. doi: 10.1073/pnas.0308485100

61. Faruque SM, Mekalanos JJ. Pathogenicity islands and phages in *Vibrio cholerae* evolution. *Trends Microbiol*. (2003) 11:505–10. doi: 10.1016/j.tim.2003.09.003

62. Chun J, Grim CJ, Hasan NA, Je HL, Seon YC, Haley BJ, et al. Comparative genomics reveals mechanism for short-term and long-term clonal transitions in pandemic *Vibrio cholerae*. *Proc Natl Acad Sci U S A*. (2009) 106:15442–7. doi: 10.1073/pnas.0907787106

63. Karaolis DKR, Lan R, Kaper JB, Reeves PR. Comparison of *vibrio cholerae* pathogenicity islands in sixth and seventh pandemic strains. *Infect Immun*. (2001) 69:1947–52. doi: 10.1128/IAI.69.3.1947

64. Boyd EF, Heilpern AJ, Waldor MK. Molecular analyses of a putative CTXφ precursor and evidence for independent acquisition of distinct CTXφs by toxigenic *Vibrio cholerae*. *J Bacteriol*. (2000) 182:5530–8. doi: 10.1128/jb.182.19.5530-5538.2000

65. Boyd EF, Moyer KE, Shi LEI, Waldor MK, Al BET. Infectious CTXPhi and the vibrio pathogenicity island prophage in Vibrio mimicus: evidence for recent horizontal transfer between V. mimicus and *V. cholerae*. *Infect Immun*. (2000) 68:1507–13. doi: 10.1128/IAI.68.3.1507-1513.2000

66. Banerjee R, Das B, Balakrish Nair G, Basak S. Dynamics in genome evolution of *Vibrio cholerae*. *Infect Genet Evol*. (2014) 23:32–41. doi: 10.1016/j.meegid.2014.01.006

67. Kim EJ, Lee D, Moon SH, Lee CH, Kim SJ, Lee JH, et al. Molecular insights into the evolutionary pathway of *Vibrio cholerae* O1 atypical el tor variants. *PLoS Pathog*. (2014) 10:e1004384. doi: 10.1371/journal.ppat.1004384

68. Waldor MK, Mekalanos JJ. Lysogenic conversion by a filamentous phage encoding. *Science*. (1996) 272:1910–4. doi: 10.1126/science.272.5270.1910

69. Korotkov KV, Sandkvist M, Hol WGJ. The type II secretion system: biogenesis, molecular architecture and mechanism. *Nat Rev Microbiol*. (2012) 10:336–51. doi: 10.1038/nrmicro2762

70. Rasti ES, Schappert ML, Brown AC. Association of *Vibrio cholerae* 569B outer membrane vesicles with host cells occurs in a GM1-independent manner. *Cell Microbiol*. (2018) 20:1–11. doi: 10.1111/cmi.12828

71. Zingl FG, Thapa HB, Scharf M, Kohl P, Müller AM, Schild S. Outer membrane vesicles of *Vibrio cholerae* protect and deliver active cholera toxin to host cells via porin-dependent uptake. *mBio*. (2021) 12:e0053421. doi: 10.1128/mBio.00534-21

72. Rasti ES, Brown AC. Cholera toxin encapsulated within several *Vibrio cholerae* O1 serotype inaba outer membrane vesicles lacks a functional B-subunit. *Toxins*. (2019) 11:1–15. doi: 10.3390/toxins11040207

73. Mathieu-Denoncourt A, Giacomucci S, Duperthuy M. "The Secretome of *Vibrio cholerae*," in *Infections and Sepsis Development*. eds. V. Neri, L. Huang and J. Li, IntechOpen.

74. Woida PJ, Satchell KJF. The *Vibrio cholerae* MARTX toxin silences the inflammatory response to cytoskeletal damage before inducing actin cytoskeleton collapse. *Sci Signal*. (2020) 13:1–16. doi: 10.1126/scisignal.aaw9447

75. Satchell KJF. Multifunctional-autoprocessing repeats-in-toxin (MARTX) toxins of vibrios. *Microbiol Spectr*. (2015) 3:VE-0002-2014. doi: 10.1128/microbiolspec.ve-0002-2014

76. Coelho A, Andrade JRC, Vicente ACP, Dirita VJ. Cytotoxic cell vacuolating activity from *Vibrio cholerae* hemolysin. *Infect Immun*. (2000) 68:1700–5. doi: 10.1128/IAI.68.3.1700-1705.2000

77. Mitra R, Figueroa P, Mukhopadhyay AK, Shimada T, Takeda Y, Berg DE, et al. Cell vacuolation, a manifestation of the El Tor hemolysin of *Vibrio cholerae*. *Infect Immun*. (2000) 68:1928–33. doi: 10.1128/IAI.68.4.1928-1933.2000

78. Sikora AE, Zielke RA, Lawrence DA, Andrews PC, Sandkvist M. Proteomic analysis of the *Vibrio cholerae* type II secretome reveals new proteins, including three related serine proteases. *J Biol Chem*. (2011) 286:16555–66. doi: 10.1074/jbc.M110.211078

79. Elluri S, Enow C, Vdovikova S, Rompikuntal PK, Dongre M, Carlsson S, et al. Outer membrane vesicles mediate transport of biologically active *Vibrio cholerae* cytolysin (VCC) from *V. cholerae* strains. *PLoS One*. (2014) 9:e106731. doi: 10.1371/journal.pone.0106731

80. Alm RA, Mayrhofer G, Kotlarski I, Manning PA. Amino-terminal domain of the El Tor haemolysin of *Vibrio cholerae* O1 is expressed in classical strains and is cytotoxic. *Vaccine*. (1991) 9:588–94. doi: 10.1016/0264-410X(91)90247-4

81. Levine MM, Kaper JB, Herrington D, Losonsky G, Morris JG, Clements ML, et al. Volunteer studies of deletion mutants of *Vibrio cholerae* O1 prepared by recombinant techniques. *Infect Immun*. (1988) 56:161–7. doi: 10.1128/iai.56.1.161-167.1988

82. Goldblum SE, Rai U, Tripathi A, Thakar M, De Leo L, Di Toro N, et al. The active zot domain (aa 288–293) increases ZO-1 and myosin 1C serine/threonine phosphorylation, alters interaction between ZO-1 and its binding partners, and induces tight junction disassembly through proteinase activated receptor 2 activation. *FASEB J*. (2011) 25:144–58. doi: 10.1096/fj.10-158972

83. Chatterjee T, Mukherjee D, Dey S, Pal A, Hoque KM, Chakrabarti P. Accessory cholera enterotoxin, ace, from *Vibrio cholerae*: structure, unfolding, and virstatin binding. *Biochemistry*. (2011) 50:2962–72. doi: 10.1021/bi101673x

84. Attridge SR, Rowley D. The role of the flagellum in the adherence of *Vibrio cholerae*. *J Infect Dis*. (1983) 147:864–72. doi: 10.1093/infdis/147.5.864

85. Richardson K. Roles of motility and flagellar structure in pathogenicity of *Vibrio cholerae*: analysis of motility mutants in three animal models. *Infect Immun*. (1991) 59:2727–36. doi: 10.1128/iai.59.8.2727-2736.1991

86. Butler SM, Camilli A. Both chemotaxis and net motility greatly influence the infectivity of *Vibrio cholerae*. *Proc Natl Acad Sci U S A*. (2004) 101:5018–23. doi: 10.1073/pnas.0308052101

87. Pennetzdorfer N, Höfler T, Wölflingseder M, Tutz S, Schild S, Reidl J. σE controlled regulation of porin OmpU in *Vibrio cholerae*. *Mol Microbiol*. (2021) 115:1244–61. doi: 10.1111/mmi.14669

88. Sakharwade SC, Sharma PK, Mukhopadhaya A. *Vibrio cholerae* Porin OmpU induces pro-inflammatory responses, but down-regulates LPS-mediated effects in RAW 264.7, THP-1 and human PBMCs. *PLoS One*. (2013) 8:e76583. doi: 10.1371/journal.pone.0076583

89. Mathur J, Waldor MK. The *Vibrio cholerae* ToxR-regulated porin OmpU confers resistance to antimicrobial peptides. *Infect Immun*. (2004) 72:3577–83. doi: 10.1128/IAI.72.6.3577-3583.2004

90. Ganie HA, Choudhary A, Baranwal S. Structure, regulation, and host interaction of outer membrane protein U (OmpU) of Vibrio species. *Microb Pathog*. (2022) 162:105267. doi: 10.1016/j.micpath.2021.105267

91. Finkelstein RA, Boesman Finkelstein M, Holt P. *Vibrio cholerae* hemagglutinin/lectin/protease hydrolyzes fibronectin and ovomucin: F.M. Burnet revisited. *Proc Natl Acad Sci U S A*. (1983) 80:1092–5. doi: 10.1073/pnas.80.4.1092

92. Wu Z, Milton D, Nybom P, Sjö A, Magnusson KE. *Vibrio cholerae* hemagglutinin/protease (HA/protease) causes morphological changes in cultured epithelial cells and perturbs their paracellular barrier function. *Microb Pathog*. (1996) 21:111–23. doi: 10.1006/mpat.1996.0047

93. Silva AJ, Pham K, Benitez JA. Haemagglutinin/protease expression and mucin gel penetration in El Tor biotype *Vibrio cholerae*. *Microbiology*. (2003) 149:1883–91. doi: 10.1099/mic.0.26086-0

94. Szabady RL, Yanta JH, Halladin DK, Schofield MJ, Welch RA. TagA is a secreted protease of *Vibrio cholerae* that specifically cleaves mucin glycoproteins. *Microbiology*. (2011) 157:516–25. doi: 10.1099/mic.0.044529-0

95. Millet YA, Alvarez D, Ringgaard S, von Andrian UH, Davis BM, Waldor MK. Insights into *Vibrio cholerae* intestinal colonization from monitoring fluorescently labeled bacteria. *PLoS Pathog*. (2014) 10:e1004405. doi: 10.1371/journal.ppat.1004405

96. Galen JE, Ketley JM, Fasano A, Richardson SH, Wasserman SS, Kaper JB. Role of *Vibrio cholerae* neuraminidase in the function of cholera toxin. *Infect Immun*. (1991) 60:406–15. doi: 10.1128/iai.60.2.406-415.1992

97. Moustafa I, Connaris H, Taylor M, Zaitsev V, Wilson JC, Kiefel MJ, et al. Sialic acid recognition by *Vibrio cholerae* neuraminidase. *J Biol Chem*. (2004) 279:40819–26. doi: 10.1074/jbc.M404965200

98. Almagro-Moreno S, Boyd EF. Sialic acid catabolism confers a competitive advantage to pathogenic *Vibrio cholerae* in the mouse intestine. *Infect Immun*. (2009) 77:3807–16. doi: 10.1128/IAI.00279-09

99. Sikora AE. Proteins secreted via the type II secretion system: smart strategies of *Vibrio cholerae* to maintain fitness in different ecological niches. *PLoS Pathog*. (2013) 9:e1003126. doi: 10.1371/journal.ppat.1003126

100. Kirn TJ, Jude BA, Taylor RK. A colonization factor links *Vibrio cholerae* environmental survival and human infection. *Nature*. (2005) 438:863–6. doi: 10.1038/nature04249

101. Wong E, Vaaje-Kolstad G, Ghosh A, Hurtado-Guerrero R, Konarev PV, Ibrahim AFM, et al. The *Vibrio cholerae* colonization factor GbpA possesses a modular structure that governs binding to different host surfaces. *PLoS Pathog*. (2012) 8:1–12. doi: 10.1371/journal.ppat.1002373

102. Syed KA, Beyhan S, Correa N, Queen J, Liu J, Peng F, et al. The *Vibrio cholerae* flagellar regulatory hierarchy controls expression of virulence factors. *J Bacteriol*. (2009) 191:6555–70. doi: 10.1128/JB.00949-09

103. Johnson TL, Fong JC, Rule C, Rogers A, Yildiz FH, Sandkvist M. The type II secretion system delivers matrix proteins for biofilm formation by *Vibrio cholerae*. *J Bacteriol*. (2014) 196:4245–52. doi: 10.1128/JB.01944-14

104. MacIntyre DL, Miyata ST, Kitaoka M, Pukatzki S. The *Vibrio cholerae* type VI secretion system displays antimicrobial properties. *Proc Natl Acad Sci U S A*. (2010) 107:19520–4. doi: 10.1073/pnas.1012931107

105. Zhao W, Caro F, Robins W, Mekalanos JJ. Antagonism toward the intestinal microbiota and its effect on *Vibrio cholerae* virulence. *Science*. (2018) 359:210–3. doi: 10.1126/science.aap8775

106. Azman AS, Rudolph KE, Cummings DAT, Lessler J, Tor E. The incubation period of cholera: a systematic review supplement. *J Infect*. (2014) 66:432–8. doi: 10.1016/j.jinf.2012.11.013.The

107. Azman AS, Rudolph KE, Cummings DAT, Lessler J. The incubation period of cholera: a systematic review. *J Infect*. (2013) 66:432–8. doi: 10.1016/j.jinf.2012.11.013

108. Scott Merrell D, Bailey C, Kaper JB, Camilli A. The ToxR-mediated organic acid tolerance response of *Vibrio cholerae* requires OmpU. *J Bacteriol*. (2001) 183:2746–54. doi: 10.1128/JB.183.9.2746-2754.2001

109. Merrell DS, Hava DL, Camilli A. Identification of novel factors involved in colonization and acid tolerance of *Vibrio cholerae*. *Mol Microbiol*. (2002) 43:1471–91. doi: 10.1046/j.1365-2958.2002.02857.x

110. Merrell DS, Camilli A. The cadA gene of *Vibrio cholerae* is induced during infection and plays a role in acid tolerance. *Mol Microbiol*. (1999) 34:836–49. doi: 10.1046/j.1365-2958.1999.01650.x

111. Sack DA, Tacket CO, Cohen MB, Sack RB, Losonsky GA, Shimko J, et al. Validation of a volunteer model of cholera with frozen bacteria as the challenge. *Infect Immun*. (1998) 66:1968–72. doi: 10.1128/iai.66.5.1968-1972.1998

112. Ramamurthy T, Nandy RK, Mukhopadhyay AK, Dutta S, Mutreja A, Okamoto K, et al. Virulence regulation and innate host response in the pathogenicity of *Vibrio cholerae*. *Front Cell Infect Microbiol*. (2020) 10:1–22. doi: 10.3389/fcimb.2020.572096

113. Childers BM, Klose KE. Regulation of virulence in *Vibrio cholerae*: the ToxR regulon. *Future Microbiol*. (2007) 2:335–44. doi: 10.2217/17460913.2.3.335

114. Zingl FG, Kohl P, Cakar F, Leitner DR, Mitterer F, Bonnington KE, et al. Outer membrane vesiculation facilitates surface exchange and in vivo adaptation of *Vibrio cholerae*. *Cell Host Microbe*. (2020) 27:225–237.e8. doi: 10.1016/j.chom.2019.12.002

115. Simonet VC, Baslé A, Klose KE, Delcour AH. The *Vibrio cholerae* porins OmpU and OmPT have distinct channel properties. *J Biol Chem*. (2003) 278:17539–45. doi: 10.1074/jbc.M301202200

116. Duret G, Delcour AH. Deoxycholic acid blocks *Vibrio cholerae* OmpT but not OmpU porin. *J Biol Chem*. (2006) 281:19899–905. doi: 10.1074/jbc.M602426200

117. Almagro-Moreno S, Pruss K, Taylor RK. Intestinal colonization dynamics of *Vibrio cholerae*. *PLoS Pathog*. (2015) 11:1–11. doi: 10.1371/journal.ppat.1004787

118. Hung DT, Mekalanos JJ. Bile acids induce cholera toxin expression in *Vibrio cholerae* in a ToxT-independent manner. *Proc Natl Acad Sci U S A*. (2005) 102:3028–33. doi: 10.1073/pnas.0409559102

119. Abuaita BH, Withey JH. Bicarbonate induces *Vibrio cholerae* virulence gene expression by enhancing ToxT activity. *Infect Immun*. (2009) 77:4111–20. doi: 10.1128/IAI.00409-09

120. Pennetzdorfer N, Lembke M, Pressler K, Matson JS, Reidl J, Schild S. Regulated proteolysis in *Vibrio cholerae* allowing rapid adaptation to stress conditions. *Front Cell Infect Microbiol*. (2019) 9:1–9. doi: 10.3389/fcimb.2019.00214

121. Atuma C, Strugala V, Allen A, Holm L. The adherent gastrointestinal mucus gel layer: thickness and physical state in vivo. *Am J Physiol Gastrointest Liver Physiol*. (2001) 280:922–9. doi: 10.1152/ajpgi.2001.280.5.g922

122. Butler SM, Camilli A. Going against the grain: chemotaxis and infection in *Vibrio cholerae*. *Nat Rev Microbiol*. (2005) 3:611–20. doi: 10.1038/nrmicro1207

123. Stewart-Tull DES, Ollar RA, Scobie TS. Studies on the *Vibrio cholerae* mucinase complex. I. Enzymic activities associated with the complex. *J Med Microbiol*. (1986) 22:325–33. doi: 10.1099/00222615-22-4-325

124. Silva AJ, Benitez JA. *Vibrio cholerae* biofilms and cholera pathogenesis. *PLoS Negl Trop Dis*. (2016) 10:1–25. doi: 10.1371/journal.pntd.0004330

125. Cho JY, Liu R, Macbeth JC, Hsiao A. The Interface of *Vibrio cholerae* and the gut microbiome. *Gut Microbes*. (2021) 13:1937015. doi: 10.1080/19490976.2021.1937015

126. Bachmann V, Kostiuk B, Unterweger D, Diaz-Satizabal L, Ogg S, Pukatzki S. Bile salts modulate the mucin-activated type VI secretion system of pandemic *Vibrio cholerae*. *PLoS Negl Trop Dis*. (2015) 9:e0004031. doi: 10.1371/journal.pntd.0004031

127. Santoriello FJ, Michel L, Unterweger D, Pukatzki S. Pandemic *Vibrio cholerae* shuts down site-specific recombination to retain an interbacterial defence mechanism. *Nat Commun*. (2020) 11:6246. doi: 10.1038/s41467-020-20012-7

128. Alavi S, Mitchell JD, Cho JY, Liu R, Macbeth JC, Hsiao A. Interpersonal gut microbiome variation drives susceptibility and resistance to cholera infection. *Cells*. (2020) 181:1533–1546.e13. doi: 10.1016/j.cell.2020.05.036

129. Jude BA, Martinez RM, Skorupski K, Taylor RK. Levels of the secreted *Vibrio cholerae* attachment factor GbpA are modulated by quorum-sensing-induced proteolysis. *J Bacteriol*. (2009) 191:6911–7. doi: 10.1128/JB.00747-09

130. Bhowmick R, Ghosal A, Das B, Koley H, Saha DR, Ganguly S, et al. Intestinal adherence of *Vibrio cholerae* involves a coordinated interaction between colonization factor GbpA and mucin. *Infect Immun*. (2008) 76:4968–77. doi: 10.1128/IAI.01615-07

131. Sperandio V, Giron JA, Silveira WD, Kaper JB. The OmpU outer membrane protein, a potential adherence factor of *Vibrio cholerae*. *Infect Immun*. (1995) 63:4433–8. doi: 10.1128/iai.63.11.4433-4438.1995

132. Yang M, Liu Z, Hughes C, Stern AM, Wang H, Zhong Z, et al. Bile salt-induced intermolecular disulfide bond formation activates *Vibrio cholerae* virulence. *Proc Natl Acad Sci U S A*. (2013) 110:2348–53. doi: 10.1073/pnas.1218039110

133. Herrington DA, Hall RH, Losonsky G, Mekalanos JJ, Taylor RK, Levine MM. Toxin, toxin-coregulated pili, and the toxR regulon are essential for *Vibrio cholerae* pathogenesis in humans. *J Exp Med*. (1988) 168:1487–92. doi: 10.1084/jem.168.4.1487

134. Kirn TJ, Lafferty MJ, Sandoe CMP, Taylor RK. Delineation of pilin domains required for bacterial association into microcolonies and intestinal colonization by *Vibrio cholerae*. *Mol Microbiol*. (2000) 35:896–910. doi: 10.1046/j.1365-2958.2000.01764.x

135. Miniewcz A, Jakubas R, Eoolivei C. Ferroelasticity of [NH2(CH3)2]3Sb2Br9 (dmaba). *Ferroelectrics*. (1990) 106:249–54. doi: 10.1080/00150199008214591

136. Zhang RG, Westbrook ML, Nance S, Spangler BD, Westbrook EM, Scott DL, et al. The three-dimensional crystal structure of cholera toxin. *J Mol Biol*. (1995) 251:563–73. doi: 10.1006/jmbi.1995.0456

137. Nours L, Rossjohn J, Beddoe T, Paton AW, Paton JC. Structure, biological functions and applications of the AB 5 toxins. *Trends Biochem Sci*. (2010) 35:411–8. doi: 10.1016/j.tibs.2010.02.003

138. Vanden DV, Horvath C, De Wolf MJ. *Vibrio cholerae*: cholera toxin. *Int J Biochem Cell Biol*. (2020) 39:1771–5. doi: 10.1016/j.biocel.2007.07.005

139. Kauffman RC, Bhuiyan TR, Nakajima R, Mayo-Smith LM, Rashu R, Hoq MR, et al. Single-cell analysis of the plasmablast response to *Vibrio cholerae* demonstrates expansion of cross-reactive memory B cells. *mBio*. (2016) 7:e02021-16. doi: 10.1128/mBio.02021-16

140. Heim JB, Hodnik V, Heggelund JE, Anderluh G. Crystal structures of cholera toxin in complex with fucosylated receptors point to importance of secondary binding site. *Sci Rep*. (2019) 9:12243. doi: 10.1038/s41598-019-48579-2

141. Pérez-Reytor D, Jaña V, Pavez L, Navarrete P, García K. Accessory toxins of Vibrio pathogens and their role in epithelial disruption during infection. *Front Microbiol*. (2018) 9:1–11. doi: 10.3389/fmicb.2018.02248

142. Nielsen AT, Dolganov NA, Otto G, Miller MC, Cheng YW, Schoolnik GK. RpoS controls the *Vibrio cholerae* mucosal escape response. *PLoS Pathog*. (2006) 2:0933–48. doi: 10.1371/journal.ppat.0020109

143. Zhu J, Miller MB, Vance RE, Dziejman M, Bassler BL, Mekalanos JJ. Quorum-sensing regulators control virulence gene expression in *Vibrio cholerae*. *Proc Natl Acad Sci U S A*. (2002) 99:3129–34. doi: 10.1073/pnas.052694299

144. Wang H, Ayala JC, Benitez JA, Silva AJ. Interaction of the histone-like nucleoid structuring protein and the general stress response regulator rpos at *Vibrio cholerae* promoters that regulate motility and hemagglutinin/protease expression. *J Bacteriol*. (2012) 194:1205–15. doi: 10.1128/JB.05900-11

145. Zhu J, Mekalanos JJ. Quorum sensing-dependent biofilms enhance colonization in *Vibrio cholerae*, the phenomenon by which bacteria monitor their cell population density through the extracellular accumulation of signaling molecules called autoinduc. *Dev Cell*. (2003) 5:647–656. doi: 10.1016/S1534-5807(03)00295-8

146. Merrell DS, Butler SM, Qadri F, Dolganov NA, Alam A, Cohen MB, et al. Host-induced epidemic spread of the cholera bacterium. *Nature*. (2002) 417:642–5. doi: 10.1038/nature00778

147. Butler SM, Nelson EJ, Chowdhury N, Faruque SM, Calderwood SB, Camilli A. Cholera stool bacteria repress chemotaxis to increase infectivity. *Mol Microbiol*. (2006) 60:417–26. doi: 10.1111/j.1365-2958.2006.05096.x

148. Nelson EJ, Chowdhury A, Flynn J, Schild S, Bourassa L, Shao Y, et al. Transmission of *Vibrio cholerae* is antagonized by lytic phage and entry into the aquatic environment. *PLoS Pathog*. (2008) 4:e1000187. doi: 10.1371/journal.ppat.1000187

149. Nelson EJ, Harris JB, Morris JG, Calderwood SB, Camilli A. Cholera transmission: the host, pathogen and bacteriophage dynamic. *Nat Rev Microbiol*. (2009) 7:693–702. doi: 10.1038/nrmicro2204

150. Benčić Z, Sinha R. Cholera carriers and circulation of cholera vibrios in the community. *Int J Epidemiol*. (1972) 1:13–4. doi: 10.1093/ije/1.1.13

151. Leung T, Matrajt L. Protection afforded by previous *Vibrio cholerae* infection against subsequent disease and infection: a review. *PLoS Negl Trop Dis*. (2021) 15:1–17. doi: 10.1371/journal.pntd.0009383

152. Naidu A, Lulu SS. Mucosal and systemic immune responses to *Vibrio cholerae* infection and oral cholera vaccines (OCVs) in humans: a systematic review. *Expert Rev Clin Immunol*. (2022) 18:1307–18. doi: 10.1080/1744666X.2022.2136650

153. Qadri F, Bhuiyan TR, Dutta KK, Raqib R, Alam MS, Alam NH. Acute dehydrating disease caused by *Vibrio cholerae* serogroups O1 and O139 induce increases in innate cells and inflammatory mediators at the mucosal surface of the gut. *Gut*. (2004) 53:62–9. doi: 10.1136/gut.53.1.62

154. Flach CF, Qadri F, Bhuiyan TR, Alam NH, Jennische E, Lönnroth I, et al. Broad up-regulation of innate defense factors during acute cholera. *Infect Immun*. (2007) 75:2343–50. doi: 10.1128/IAI.01900-06

155. Mathan MM, Chandy G, Mathan VI. Ultrastructural changes in the upper small intestinal mucosa in patients with cholera. *Gastroenterology*. (1995) 109:422–30. doi: 10.1016/0016-5085(95)90329-1

156. Peterson LW, Artis D. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. *Nat Rev Immunol*. (2014) 14:141–53. doi: 10.1038/nri3608

157. Bandyopadhaya A, Sarkar M, Chaudhuri K. Transcriptional upregulation of inflammatory cytokines in human intestinal epithelial cells following *Vibrio cholerae* infection. *FEBS J*. (2007) 274:4631–42. doi: 10.1111/j.1742-4658.2007.05991.x

158. Rodríguez BL, Rojas A, Campos J, Ledon T, Valle E, Toledo W, et al. Differential interleukin-8 response of intestinal epithelial cell line to reactogenic and nonreactogenic candidate vaccine strains of *Vibrio cholerae*. *Infect Immun*. (2001) 69:613–6. doi: 10.1128/IAI.69.1.613-616.2001

159. Harrison LM, Rallabhandi P, Michalski J, Zhou X, Steyert SR, Vogel SN, et al. *Vibrio cholerae* flagellins induce toll-like receptor 5-mediated interleukin-8 production through mitogen-activated protein kinase and NF-κB activation. *Infect Immun*. (2008) 76:5524–34. doi: 10.1128/IAI.00843-08

160. Bourque DL, Bhuiyan TR, Genereux DP, Rashu R, Ellis CN, Chowdhury F, et al. Analysis of the human mucosal response to cholera reveals sustained activation of innate immune signaling pathways. *Infect Immun*. (2018) 86:e00594-17. doi: 10.1128/IAI.00594-17

161. Sarkar M, Chaudhuri K. Association of adherence and motility in interleukin 8 induction in human intestinal epithelial cells by *Vibrio cholerae*. *Microbes Infect*. (2004) 6:676–85. doi: 10.1016/j.micinf.2004.02.018

162. Ellis CN, LaRocque RC, Uddin T, Krastins B, Mayo-Smith LM, Sarracino D, et al. Comparative proteomic analysis reveals activation of mucosal innate immune signaling pathways during cholera. *Infect Immun*. (2015) 83:1089–103. doi: 10.1128/IAI.02765-14

163. Bandyopadhaya A, Sarkar M, Chaudhuri K. Human intestinal epithelial cell cytokine mRNA responses mediated by NF-κB are modulated by the motility and adhesion process of *Vibrio cholerae*. *Int J Biochem Cell Biol*. (2007) 39:1863–76. doi: 10.1016/j.biocel.2007.05.005

164. Yang JS, Jeon JH, Jang MS, Kang SS, Ahn KB, Song M, et al. *Vibrio cholerae* OmpU induces IL-8 expression in human intestinal epithelial cells. *Mol Immunol*. (2018) 93:47–54. doi: 10.1016/j.molimm.2017.11.005

165. McGee DW, Elson CO, McGhee JR. Enhancing effect of cholera toxin on interleukin-6 secretion by IEC-6 intestinal epithelial cells: mode of action and augmenting effect of inflammatory cytokines. *Infect Immun*. (1993) 61:4637–44. doi: 10.1128/iai.61.11.4637-4644.1993

166. Sitaraman SV, Merlin D, Wang L, Wong M, Gewirtz AT, Si-Tahar M, et al. Neutrophil-epithelial crosstalk at the intestinal lumenal surface mediated by reciprocal secretion of adenosine and IL-6. *J Clin Invest*. (2001) 107:861–9. doi: 10.1172/JCI11783

167. Taub DD, Anver M, Oppenheim JJ, Longo DL, Murphy WJ. T lymphocyte recruitment by interleukin-8 (IL-8): IL-8-induced degranulation of neutrophils releases potent chemoattractants for human T lymphocytes both in vitro and in vivo. *J Clin Invest*. (1996) 97:1931–41. doi: 10.1172/JCI118625

168. Remick DG. Interleukin-8. *Crit Care Med*. (2005) 33:466–7. doi: 10.1097/01.CCM.0000186783.34908.18

169. Carr MW, Roth SJ, Luther E, Rose SS, Springer TA. Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant. *Proc Natl Acad Sci U S A*. (1994) 91:3652–6. doi: 10.1073/pnas.91.9.3652

170. Xu LL, Warren MK, Rose WL, Gong W, Wang JM. Human recombinant monocyte chemotactic protein and other c-c chemokines bind and induce directional migration of dendritic cells in vitro. *J Leukoc Biol*. (1996) 60:365–71. doi: 10.1002/jlb.60.3.365

171. Pulimood AB, Ramakrishna BS, Rita AB, Srinivasan P, Mohan V, Gupta S, et al. Early activation of mucosal dendritic cells and macrophages in acute Campylobacter colitis and cholera: an in vivo study. *J Gastroenterol Hepatol*. (2008) 23:752–8. doi: 10.1111/j.1440-1746.2008.05325.x

172. Bhuiyan TR, Rahman MA, Trivedi S, Afroz T, Al Banna H, Hoq MR, et al. Mucosal-associated invariant T (MAIT) cells are highly activated in duodenal tissue of humans with *Vibrio cholerae* O1 infection: a preliminary report. *PLoS Negl Trop Dis*. (2022) 16:e0010411. doi: 10.1371/journal.pntd.0010411

173. Jensen O, Trivedi S, Meier JD, Fairfax KC, Hale JS, Leung DT. A subset of follicular helper-like MAIT cells can provide B cell help and support antibody production in the mucosa. *Sci Immunol*. (2022) 7:1–14. doi: 10.1126/sciimmunol.abe8931

174. Qadri F, Raqib R, Ahmed F, Rahman T, Wenneras C, Das SK, et al. Increased levels of inflammatory mediators in children and adults infected with *Vibrio cholerae* O1 and O139. *Clin Diagn Lab Immunol*. (2002) 9:221–9. doi: 10.1128/CDLI.9.2.221-229.2002

175. Janoff EN, Hayakawa H, Taylor DN, Fasching CE, Kenner JR, Jaimes E, et al. Nitric oxide production during *Vibrio cholerae* infection. *Am J Physiol Gastrointest Liver Physiol*. (1997) 273:1160–7. doi: 10.1152/ajpgi.1997.273.5.g1160

176. Arnold RR, Cole MF, McGhee JR. A bactericidal effect for human lactoferrin. *Science*. (1977) 197:263–5. doi: 10.1126/science.327545

177. Arnold RR, Brewer M, Gauthier JJ. Bactericidal activity of human lactoferrin: sensitivity of a variety of microorganisms. *Infect Immun*. (1980) 28:893–8. doi: 10.1128/iai.28.3.893-898.1980

178. Queen J, Fullner Satchell KJ. Neutrophils are essential for containment of *Vibrio cholerae* to the intestine during the proinflammatory phase of infection. *Infect Immun*. (2012) 80:2905–13. doi: 10.1128/IAI.00356-12

179. Dolores J, Satchell KJF. Analysis of *Vibrio cholerae* genome sequences reveals unique rtxA variants in environmental strains and an rtxA-null mutation in recent altered EL tor isolates. *mBio*. (2013) 4:e00624-12. doi: 10.1128/mBio.00624-12

180. Mörbe UM, Jørgensen PB, Fenton TM, von Burg N, Riis LB, Spencer J, et al. Human gut-associated lymphoid tissues (GALT); diversity, structure, and function. *Mucosal Immunol*. (2021) 14:793–802. doi: 10.1038/s41385-021-00389-4

181. Sheridan BS, Lefrançois L. Regional and mucosal memory T cells. *Nat Immunol*. (2011) 12:485–91. doi: 10.1038/ni.2029

182. Owen RL, Pierce NF, Apple RT, Cray WC. M cell transport of *Vibrio cholerae* from the intestinal lumen into Peyer's patches: a mechanism for antigen sampling and for microbial transepithelial migration. *J Infect Dis*. (1986) 153:1108–18. doi: 10.1093/infdis/153.6.1108

183. Kim MS, Yi EJ, Kim YI, Kim SH, Jung YS, Kim SR, et al. ERDj5 in innate immune cells is a crucial factor for the mucosal adjuvanticity of cholera toxin. *Front Immunol*. (2019) 10:1249. doi: 10.3389/fimmu.2019.01249

184. Gagliardi MC, Sallusto F, Marinaro M, Langenkamp A, Lanzavecchia A, De Magistris MT. Cholera toxin induces maturation of human dendritic cells and licences them for Th2 priming. *Eur J Immunol*. (2000) 30:2394–403. doi: 10.1002/1521-4141(2000)30:8<2394::AID-IMMU2394>3.0.CO;2-Y

185. Lavelle EC, McNeela E, Armstrong ME, Leavy O, Higgins SC, Mills KHG. Cholera toxin promotes the induction of regulatory T cells specific for bystander antigens by modulating dendritic cell activation. *J Immunol*. (2003) 171:2384–92. doi: 10.4049/jimmunol.171.5.2384

186. Datta SK, Sabet M, Nguyen KPL, Valdez PA, Gonzalez-Navajas JM, Islam S, et al. Mucosal adjuvant activity of cholera toxin requires Th17 cells and protects against inhalation anthrax. *Proc Natl Acad Sci U S A*. (2010) 107:10638–43. doi: 10.1073/pnas.1002348107

187. Kimura A, Kishimoto T. IL-6: regulator of Treg/Th17 balance. *Eur J Immunol*. (2010) 40:1830–5. doi: 10.1002/eji.201040391

188. Shreedhar VK, Kelsall BL, Neutra MR. Cholera toxin induces migration of dendritic cells from the subepithelial dome region to T- and B-cell areas of Peyer's patches. *Infect Immun*. (2003) 71:504–9. doi: 10.1128/IAI.71.1.504-509.2003

189. Weil AA, Ellis CN, Debela MD, Bhuiyan TR, Rashu R, Bourque DL, et al. Posttranslational regulation of IL-23 production distinguishes the innate immune responses to live toxigenic versus heat-inactivated *Vibrio cholerae*. *mSphere*. (2019) 4:e00206-19. doi: 10.1128/msphere.00206-19

190. Zughaier SM, Zimmer SM, Datta A, Carlson RW, Stephens DS. Differential induction of the toll-like receptor 4-MyD88-dependent and -independent signaling pathways by endotoxins. *Infect Immun*. (2005) 73:2940–50. doi: 10.1128/IAI.73.5.2940-2950.2005

191. Khader SA, Gaffen SL, Kolls JK. Th17 cells at the crossroads of innate and adaptive immunity against infectious diseases at the mucosa. *Mucosal Immunol*. (2009) 2:403–11. doi: 10.1038/mi.2009.100

192. Volpe E, Servant N, Zollinger R, Bogiatzi SI, Hupé P, Barillot E, et al. A critical function for transforming growth factor-β, interleukin 23 and proinflammatory cytokines in driving and modulating human TH-17 responses. *Nat Immunol*. (2008) 9:650–7. doi: 10.1038/ni.1613

193. Kuchta A, Rahman T, Sennott EL, Bhuyian TR, Uddin T, Rashu R, et al. *Vibrio cholerae* O1 infection induces proinflammatory CD4 + T-cell responses in blood and intestinal mucosa of infected humans. *Clin Vaccine Immunol*. (2011) 18:1371–7. doi: 10.1128/CVI.05088-11

194. Bhuiyan TR, Lundin SB, Khan AI, Lundgren A, Harris JB, Calderwood SB, et al. Cholera caused by *Vibrio cholerae* O1 induces T-cell responses in the circulation. *Infect Immun*. (2009) 77:1888–93. doi: 10.1128/IAI.01101-08

195. Qadri F, Clemens JD, Holmgren J. Cholera immunity and development and use of oral cholera vaccines for disease control In: H Kiyono and DW Pascual, editors. *Mucosal vaccines. 2nd* ed. Amsterdam: Elsevier Inc (2019). 537–61.

196. Crotty S. T follicular helper cell differentiation, function, and roles in disease. *Immunity*. (2014) 41:529–42. doi: 10.1016/j.immuni.2014.10.004

197. Rashu R, Bhuiyan TR, Hoq MR, Hossain L, Paul A, Khan AI, et al. Cognate T and B cell interaction and association of follicular helper T cells with B cell responses in *Vibrio cholerae* O1 infected Bangladeshi adults. *Microbes Infect*. (2019) 21:176–83. doi: 10.1016/j.micinf.2018.12.002

198. Charles RC, Nakajima R, Liang L, Jasinskas A, Berger A, Leung DT, et al. Plasma and mucosal immunoglobulin M, immunoglobulin a, and immunoglobulin G responses to the *Vibrio cholerae* O1 protein immunome in adults with cholera in Bangladesh. *J Infect Dis*. (2017) 216:125–34. doi: 10.1093/infdis/jix253

199. Mayo-Smith LM, Simon JK, Chen WH, Haney D, Lock M, Lyon CE, et al. The live attenuated cholera vaccine CVD 103-HgR primes responses to the toxin-coregulated pilus antigen TcpA in subjects challenged with wild-type *Vibrio cholerae*. *Clin Vaccine Immunol*. (2017) 24:e00470-16. doi: 10.1128/CVI.00470-16

200. Harris AM, Bhuiyan MS, Chowdhury F, Khan AI, Hossain A, Kendall EA, et al. Antigen-specific memory B-cell responses to *Vibrio cholerae* O1 infection in Bangladesh. *Infect Immun*. (2009) 77:3850–6. doi: 10.1128/IAI.00369-09

201. Arifuzzaman M, Rashu R, Leung DT, Hosen MI, Bhuiyan TR, Bhuiyan MS, et al. Antigen-specific memory T cell responses after vaccination with an oral killed cholera vaccine in Bangladeshi children and comparison to responses in patients with naturally acquired cholera. *Clin Vaccine Immunol*. (2012) 19:1304–11. doi: 10.1128/CVI.00196-12

202. Weil AA, Becker RL, Harris JB. *Vibrio cholerae* at the intersection of immunity and the microbiome. *mSphere*. (2019) :e00597-19. doi: 10.1128/msphere.00597-19

203. Weil AA, Arifuzzaman M, Bhuiyan TR, LaRocque RC, Harris AM, Kendall EA, et al. Memory T-cell responses to *Vibrio cholerae* O1 infection. *Infect Immun*. (2009) 77:5090–6. doi: 10.1128/IAI.00793-09

204. Chowdhury F, Khan AI, Harris JB, Larocque RC, Chowdhury MI, Ryan ET, et al. A comparison of clinical and immunologic features in children and older patients hospitalized with severe cholera in Bangladesh. *Pediatr Infect Dis J*. (2008) 27:986–92. doi: 10.1097/INF.0b013e3181783adf

205. Aktar A, Rahman MA, Afrin S, Akter A, Uddin T, Yasmin T, et al. Plasma and memory B cell responses targeting O-specific polysaccharide (OSP) are associated with protection against *Vibrio cholerae* O1 infection among household contacts of cholera patients in Bangladesh. *PLoS Negl Trop Dis*. (2018) 12:1–15. doi: 10.1371/journal.pntd.0006399

206. Harris JB. Cholera: immunity and prospects in vaccine development. *J Infect Dis*. (2018) 218:S141–6. doi: 10.1093/infdis/jiy414

207. Saha D, LaRocque RC, Khan AI, Harris JB, Begum YA, Akramuzzaman SM, et al. Incomplete correlation of serum vibriocidal antibody titer with protection from *Vibrio cholerae* infection in urban Bangladesh. *J Infect Dis*. (2004) 189:2318–22. doi: 10.1086/421275

208. Haney DJ, Lock MD, Simon JK, Harris J, Gurwith M. Antibody-based correlates of protection against cholera: analysis of a challenge study of a cholera-naive population. *Clin Vaccine Immunol*. (2017) 24:1–11. doi: 10.1128/CVI.00098-17

209. Chen K, Magri G, Grasset EK, Cerutti A. Rethinking mucosal antibody responses: IgM, IgG and IgD join IgA. *Nat Rev Immunol*. (2020) 20:427–41. doi: 10.1038/s41577-019-0261-1

210. Kamada N, Sakamoto K, Seo S-U, Zeng MY, Kim Y-G, Cascalho M, et al. Humoral immunity in the gut selectively targets phenotypically virulent attaching-and-effacing Bacteria for intraluminal elimination. *Cell Host Microbe*. (2015) 17:617–27. doi: 10.1016/j.chom.2015.04.001

211. Charles RC, Kelly M, Tam JM, Akter A, Hossain M, Islam K, et al. Humans surviving cholera develop antibodies against *Vibrio cholerae* O-specific polysaccharide that inhibit pathogen motility. *MBio*. (2020) 11:1–13. doi: 10.1128/mBio.02847-20

212. Apter FM, Michetti P, Winner LS, Mack JA, Mekalanos JJ, Neutra MR. Analysis of the roles of antilipopolysaccharide and anti-cholera toxin immunoglobulin A (IgA) antibodies in protection against *Vibrio cholerae* and cholera toxin by use of monoclonal IgA antibodies in vivo. *Infect Immun*. (1993) 61:5279–85. doi: 10.1128/iai.61.12.5279-5285.1993

213. Patel SM, Rahman MA, Mohasin M, Riyadh MA, Leung DT, Alam MM, et al. Memory B cell responses to *Vibrio cholerae* O1 lipopolysaccharide are associated with protection against infection from household contacts of patients with cholera in Bangladesh. *Clin Vaccine Immunol*. (2012) 19:842–8. doi: 10.1128/CVI.00037-12

214. Kaisar MH, Bhuiyan MS, Akter A, Saleem D, Iyer AS, Dash P, et al. *Vibrio cholerae* sialidase-specific immune responses are associated with protection against cholera. *mSphere*. (2021) 6:e01232-20. doi: 10.1128/msphere.01232-20

215. Holmgren J. Modern history of cholera vaccines and the pivotal role of icddr,b. *J Infect Dis*. (2021) 224:S742–8. doi: 10.1093/infdis/jiab423

216. Czerkinsky C, Holmgren J. Mucosal delivery routes for optimal immunization: targeting immunity to the right tissues In: P Kozlowski, editor. *Mucosal vaccines. Current topics in microbiology and immunology*. Berlin, Heidelberg: Springer (2010)

217. Davitt CJH, Lavelle EC. Delivery strategies to enhance oral vaccination against enteric infections. *Adv Drug Deliv Rev*. (2015) 91:52–69. doi: 10.1016/j.addr.2015.03.007

218. Ali M, Emch M, Von Seidlein L, Yunus M, Sack DA, Rao M, et al. Herd immunity conferred by killed oral cholera vaccines in Bangladesh: a reanalysis. *Lancet*. (2005) 366:44–9. doi: 10.1016/S0140-6736(05)66550-6

219. Ali M, Kim P, Zaman K, Clemens J. Herd protection of unvaccinated adults by oral cholera vaccines in rural Bangladesh. *Int Health*. (2019) 11:229–34. doi: 10.1093/inthealth/ihy085

220. Clemens JD, Sack DA, Harris JR, van Loon F, Chakraborty J, Ahmed F, et al. Field trial of oral cholera vaccines in Bangladesh: results from three-year follow-up. *Lancet*. (1990) 335:270–3. doi: 10.1016/0140-6736(90)90080-O

221. Sanchez JL, Vasquez B, Begue RE, Meza R, Castellares G, Cabezas C, et al. Protective efficacy of oral whole-cell/recombinant-B-subunit cholera vaccine in Peruvian military recruits. *Lancet*. (1994) 344:1273–6. doi: 10.1016/S0140-6736(94)90755-2

222. Lucas MES, Deen JL, von Seidlein L, Wang X-Y, Ampuero J, Puri M, et al. Effectiveness of mass oral cholera vaccination in Beira, Mozambique. *N Engl J Med*. (2005) 352:757–67. doi: 10.1056/NEJMoa043323

223. Quiding M, Nordström I, Kilander A, Andersson G, Hanson LÅ, Holmgren J, et al. Intestinal immune responses in humans: Oral cholera vaccination induces strong intestinal antibody responses and interferon-γ production and evokes local immunological memory. *J Clin Invest*. (1991) 88:143–8. doi: 10.1172/JCI115270

224. Clemens JD, Sack DA, Harris JR, Chakraborty J, Neogy PK, Stanton B, et al. Cross-protection by B subunit-whole cell cholera vaccine against diarrhea associated with heat-labile toxin-producing enterotoxigenic *Escherichia coli*: results of a large-scale field trial. *J Infect Dis*. (1988) 158:372–7. doi: 10.1093/infdis/158.2.372

225. Jertborn M, Svennerholm AM, Holmgren J. Intestinal and systemic imune responses in humans after oral immunization with a bivalent B subunit-O1/O139 whole cell cholera vaccine. *Vaccine*. (1996) 14:1459–65. doi: 10.1016/S0264-410X(96)00071-0

226. Shamsuzzaman S, Ahmed T, Mannoor K, Begum YA, Bardhan PK, Sack RB, et al. Robust gut associated vaccine-specific antibody-secreting cell responses are detected at the mucosal surface of Bangladeshi subjects after immunization with an oral killed bivalent *V. cholerae* O1/O139 whole cell cholera vaccine: comparison with other muco. *Vaccine*. (2009) 27:1386–92. doi: 10.1016/j.vaccine.2008.12.041

227. Holmgren J. An update on cholera immunity and current and future cholera vaccines. *Trop Med Infect Dis*. (2021) 6:64. doi: 10.3390/tropicalmed6020064

228. Bhattacharya SK, Sur D, Ali M, Kanungo S, You YA, Manna B, et al. 5 year efficacy of a bivalent killed whole-cell oral cholera vaccine in Kolkata, India: a cluster-randomised, double-blind, placebo-controlled trial. *Lancet Infect Dis*. (2013) 13:1050–6. doi: 10.1016/S1473-3099(13)70273-1

229. Sur D, Lopez AL, Kanungo S, Paisley A, Manna B, Ali M, et al. Efficacy and safety of a modified killed-whole-cell oral cholera vaccine in India: an interim analysis of a cluster-randomised, double-blind, placebo-controlled trial. *Lancet*. (2009) 374:1694–702. doi: 10.1016/S0140-6736(09)61297-6

230. Odevall L, Hong D, Digilio L, Sahastrabuddhe S, Mogasale V, Baik Y, et al. The Euvichol story – development and licensure of a safe, effective and affordable oral cholera vaccine through global public private partnerships. *Vaccine*. (2018) 36:6606–14. doi: 10.1016/j.vaccine.2018.09.026

231. Chen Q, Yu S, Wang Y. Community trial for safety and immunogenicity of oral-administered lyophilized rBS-WC cholera vaccine. *Zhonghua Yu Fang Yi Xue Za Zhi*. (1996) 30:330–3.

232. Chowdhury F, Akter A, Bhuiyan TR, Tauheed I, Teshome S, Sil A, et al. A non-inferiority trial comparing two killed, whole cell, oral cholera vaccines (Cholvax vs. Shanchol) in Dhaka, Bangladesh. *Vaccine*. (2022) 40:640–9. doi: 10.1016/j.vaccine.2021.12.015

233. Deen J, Clemens JD. Licensed and recommended inactivated Oral CholeraVaccines: from development to innovative deployment. *Trop Med Infect Dis*. (2021) 6:32. doi: 10.3390/tropicalmed6010032

234. Qadri F, Wierzba TF, Ali M, Chowdhury F, Khan AI, Saha A, et al. Efficacy of a single-dose, inactivated oral cholera vaccine in Bangladesh. *N Engl J Med*. (2016) 374:1723–32. doi: 10.1056/nejmoa1510330

235. Qadri F, Ali M, Lynch J, Chowdhury F, Khan AI, Wierzba TF, et al. Efficacy of a single-dose regimen of inactivated whole-cell oral cholera vaccine: results from 2 years of follow-up of a randomised trial. *Lancet Infect Dis*. (2018) 18:666–74. doi: 10.1016/S1473-3099(18)30108-7

236. Hauke CA, Taylor RK. Production of putative enhanced oral cholera vaccine strains that express toxin-coregulated pilus. *PLoS One*. (2017) 12:e0175170. doi: 10.1371/journal.pone.0175170

237. Di Tommaso A, De Magistris MT, Bugnoli M, Marsili I, Rappuoli R, Abrignani S. Formaldehyde treatment of proteins can constrain presentation to T cells by limiting antigen processing. *Infect Immun*. (1994) 62:1830–4. doi: 10.1128/iai.62.5.1830-1834.1994

238. McCarty J, Bedell L, De Lame P-A, Cassie D, Lock M, Bennett S, et al. Update on CVD 103-HgR single-dose, live oral cholera vaccine. *Expert Rev Vaccines*. (2022) 21:9–23. doi: 10.1080/14760584.2022.2003709

239. Levine MM, Chen WH, Kaper JB, Lock M, Danzig L, Gurwith M. PaxVax CVD 103-HgR single-dose live oral cholera vaccine. *Expert Rev Vaccines*. (2017) 16:197–213. doi: 10.1080/14760584.2017.1291348

240. Tacket CO, Cohen MB, Wasserman SS, Losonsky G, Livio S, Kotloff K, et al. Randomized, double-blind, placebo-controlled, multicentered trial of the efficacy of a single dose of live oral cholera vaccine CVD 103-HgR in preventing cholera following challenge with *Vibrio cholerae* O1 El Tor Inaba three months after vaccination. *Infect Immun*. (1999) 67:6341–5. doi: 10.1128/iai.67.12.6341-6345.1999

241. McCarty JM, Gierman EC, Bedell L, Lock MD, Bennett S. Safety and immunogenicity of live oral cholera vaccine CVD 103-HgR in children and adolescents aged 6-17 years. *Am J Trop Med Hyg*. (2020) 102:48–57. doi: 10.4269/ajtmh.19-0241

242. Richie E, Punjabi NH, Sidharta Y, Peetosutan K, Sukandar M, Wasserman SS, et al. Efficacy trial of single-dose live oral cholera vaccine CVD 103-HgR in North Jakarta, Indonesia, a cholera-endemic area. *Vaccine*. (2000) 18:2399–410. doi: 10.1016/S0264-410X(00)00006-2

243. Calain P, Chaine JP, Johnson E, Lou HM, O'Leary MJ, Oshitani H, et al. Can oral cholera vaccination play a role in controlling a cholera outbreak? *Vaccine*. (2004) 22:2444–51. doi: 10.1016/j.vaccine.2003.11.070

244. Song KR, Lim JK, Park SE, Saluja T, Il CS, Wartel TA, et al. Oral cholera vaccine efficacy and effectiveness. *Vaccine*. (2021) 9:1482. doi: 10.3390/vaccines9121482

245. Sharma T, Joshi N, Kumar Mandyal A, Nordqvist SL, Lebens M, Kanchan V, et al. Development of Hillchol®, a low-cost inactivated single strain Hikojima oral cholera vaccine. *Vaccine*. (2020) 38:7998–8009. doi: 10.1016/j.vaccine.2020.10.043

246. Chowdhury F, Ali Syed K, Akter A, Rahman Bhuiyan T, Tauheed I, Khaton F, et al. A phase I/II study to evaluate safety, tolerability and immunogenicity of Hillchol®, an inactivated single Hikojima strain based oral cholera vaccine, in a sequentially age descending population in Bangladesh. *Vaccine*. (2021) 39:4450–7. doi: 10.1016/j.vaccine.2021.06.069

247. Taylor DN, Killeen KP, Hack DC, Kenner JR, Coster TS, Beattie DT, et al. Development of a live, oral, attenuated vaccine against el tor cholera. *J Infect Dis*. (1994) 170:1518–23. doi: 10.1093/infdis/170.6.1518

248. Kenner JR, Coster TS, Taylor DN, Trofa AF, Barrera-Oro M, Hyman T, et al. Peru-15, an improved live attenuated oral vaccine candidate for *Vibrio cholerae* O1. *J Infect Dis*. (1995) 172:1126–9. doi: 10.1093/infdis/172.4.1126

249. Sack DA, Sack RB, Shimko J, Gomes G, O'Sullivan D, Metcalfe K, et al. Evaluation of Peru-15, a new live oral vaccine for cholera, in volunteers. *J Infect Dis*. (1997) 176:201–5. doi: 10.1086/514025

250. Qadri F, Chowdhury MI, Faruque SM, Salam MA, Ahmed T, Begum YA, et al. Randomized, controlled study of the safety and immunogenicity of Peru-15, a live attenuated oral vaccine candidate for cholera, in adult volunteers in Bangladesh. *J Infect Dis*. (2005) 192:573–9. doi: 10.1086/432074

251. Qadri F, Chowdhury MI, Faruque SM, Salam MA, Ahmed T, Begum YA, et al. Peru-15, a live attenuated oral cholera vaccine, is safe and immunogenic in Bangladeshi toddlers and infants. *Vaccine*. (2007) 25:231–8. doi: 10.1016/j.vaccine.2006.08.031

252. Ratanasuwan W, Kim YH, Sah BK, Suwanagool S, Kim DR, Anekthananon A, et al. Peru-15 (Choleragarde®), a live attenuated oral cholera vaccine, is safe and immunogenic in human immunodeficiency virus (HIV)-seropositive adults in Thailand. *Vaccine*. (2015) 33:4820–6. doi: 10.1016/j.vaccine.2015.07.073

253. Cohen MB, Giannella RA, Bean J, Taylor DN, Parker S, Hoeper A, et al. Randomized, controlled human challenge study of the safety, immunogenicity, and protective efficacy of a single dose of Peru-15, a live attenuated oral cholera vaccine. *Infect Immun*. (2002) 70:1965–70. doi: 10.1128/IAI.70.4.1965-1970.2002

254. Benítez JA, García L, Silva A, García H, Fando R, Cedré B, et al. Preliminary assessment of the safety and immunogenicity of a new CTXΦ- negative, hemagglutinin/protease-defective El Tor strain as a cholera vaccine candidate. *Infect Immun*. (1999) 67:539–45. doi: 10.1128/iai.67.2.539-545.1999

255. Valera R, García HM, Díaz Jidy M, Mirabal M, Armesto MI, Fando R, et al. Randomized, double-blind, placebo-controlled trial to evaluate the safety and immunogenicity of live oral cholera vaccine 638 in Cuban adults. *Vaccine*. (2009) 27:6564–9. doi: 10.1016/j.vaccine.2009.08.042

256. García HM, Thompson R, Valera R, Fando R, Fumane J, Jani I, et al. A single dose of live-attenuated 638 *Vibrio cholerae* oral vaccine is safe and immunogenic in adult volunteers in Mozambique. *VacciMonitor*. (2011) 20:1–8.

257. García L, Jidy MD, García H, Rodríguez BL, Fernández R, Año G, et al. The vaccine candidate *Vibrio cholerae* 638 is protective against cholera in healthy volunteers. *Infect Immun*. (2005) 73:3018–24. doi: 10.1128/IAI.73.5.3018-3024.2005

258. Thungapathra M, Sharma C, Gupta N, Ghosh RK, Mukhopadhyay A, Koley H, et al. Construction of a recombinant live oral vaccine from a non-toxigenic strain of *Vibrio cholerae* O1 serotype Inaba biotype El Tor and assessment of its reactogenicity and immunogenicity in the rabbit model. *Immunol Lett*. (1999) 68:219–27. doi: 10.1016/S0165-2478(99)00076-0

259. Mahalanabis D, Ramamurthy T, Nair GB, Ghosh A, Shaikh S, Sen B, et al. Randomized placebo controlled human volunteer trial of a live oral cholera vaccine VA1.3 for safety and immune response. *Vaccine*. (2009) 27:4850–6. doi: 10.1016/j.vaccine.2009.05.065

260. Kanungo S, Sen B, Ramamurthy T, Sur D, Manna B, Pazhani GP, et al. Safety and immunogenicity of a live oral recombinant cholera vaccine VA1.4: a randomized, placebo controlled trial in healthy adults in a cholera endemic area in Kolkata, India. *PLoS One*. (2014) 9:e99381. doi: 10.1371/journal.pone.0099381

261. Hubbard TP, Billings G, Dörr T, Sit B, Warr AR, Kuehl CJ, et al. A live vaccine rapidly protects against cholera in an infant rabbit model. *Sci Transl Med*. (2018) 10:1–11. doi: 10.1126/scitranslmed.aap8423

262. Sit B, Zhang T, Fakoya B, Akter A, Biswas R, Ryan ET, et al. Oral immunization with a probiotic cholera vaccine induces broad protective immunity against *Vibrio cholerae* colonization and disease in mice. *PLoS Negl Trop Dis*. (2019) 13:e0007417. doi: 10.1371/journal.pntd.0007417

263. Gupta RK, Szu SC, Finkelstein RA, Robbins JB. Synthesis, characterization, and some immunological properties of conjugates composed of the detoxified lipopolysaccharide of *Vibrio cholerae* O1 serotype inaba bound to cholera toxin. *Infect Immun*. (1992) 60:3201–8. doi: 10.1128/iai.60.8.3201-3208.1992

264. Gupta RK, Taylor DN, Bryla DA, Robbins JB, Szu SC. Phase 1 evaluation of *Vibrio cholerae* O1, serotype Inaba, polysaccharide-cholera toxin conjugates in adult volunteers. *Infect Immun*. (1998) 66:3095–9. doi: 10.1128/iai.66.7.3095-3099.1998

265. Alam MM, Bufano MK, Xu P, Kalsy A, Yu Y, Freeman YW, et al. Evaluation in mice of a conjugate vaccine for cholera made from *Vibrio cholerae* O1 (Ogawa) O-specific polysaccharide. *PLoS Negl Trop Dis*. (2014) 8:e2683. doi: 10.1371/journal.pntd.0002683

266. Sayeed MA, Bufano MK, Xu P, Eckhoff G, Charles RC, Alam MM, et al. A cholera conjugate vaccine containing ospecific polysaccharide (OSP) of *V. cholerae* O1 inaba and recombinant fragment of tetanus toxin heavy chain (OSP:rTTHC) induces serum, memory and lamina proprial responses against OSP and is protective in mice. *PLoS Negl Trop Dis*. (2015) 9:e0003881. doi: 10.1371/journal.pntd.0003881

267. Akter A, Kelly M, Charles RC, Harris JB, Calderwood SB, Bhuiyan TR, et al. Parenteral vaccination with a cholera conjugate vaccine boosts vibriocidal and anti-osp responses in mice previously immunized with an oral cholera vaccine. *Am J Trop Med Hyg*. (2021) 104:2024–30. doi: 10.4269/ajtmh.20-1511

268. Jeon S, Kelly M, Yun J, Lee B, Park M, Whang Y, et al. Scalable production and immunogenicity of a cholera conjugate vaccine. *Vaccine*. (2021) 39:6936–46. doi: 10.1016/j.vaccine.2021.10.005

269. Azegami T, Itoh H, Kiyono H, Yuki Y. Novel transgenic rice-based vaccines. *Arch Immunol Ther Exp*. (2015) 63:87–99. doi: 10.1007/s00005-014-0303-0

270. Gupta P, Andankar I, Gunasekaran B, Easwaran N, Kodiveri MG. Genetically modified potato and rice based edible vaccines – an overview. *Biocatal Agric Biotechnol*. (2022) 43:102405. doi: 10.1016/j.bcab.2022.102405

271. Salyaev RK, Rigano MM, Rekoslavskaya NI. Development of plant-based mucosal vaccines against widespread infectious diseases. *Expert Rev Vaccines*. (2010) 9:937–46. doi: 10.1586/erv.10.81

272. Arakawa T, Chong DKX, Lawrence Merritt J, Langridge WHR. Expression of cholera toxin B subunit oligomers in transgenic potato plants. *Transgenic Res*. (1997) 6:403–13. doi: 10.1023/A:1018487401810

273. Jani D, Meena LS, Rizwan-ul-Haq QM, Singh Y, Sharma AK, Tyagi AK. Expression of cholera toxin B subunit in transgenic tomato plants. *Transgenic Res*. (2002) 11:447–54. doi: 10.1023/A:1020336332392

274. Loc NH, Thinh LT, Yang MS, Kim TG. Highly expressed cholera toxin B subunit in the fruit of a transgenic tomato (*Lycopersicon esculentum* L.). *Biotechnol Bioprocess Eng*. (2011) 16:576–80. doi: 10.1007/s12257-010-0195-8

275. Nochi T, Takagi H, Yuki Y, Yang L, Masumura T, Mejima M, et al. Rice-based mucosal vaccine as a global strategy for cold-chain- and needle-free vaccination. *Proc Natl Acad Sci U S A*. (2007) 104:10986–91. doi: 10.1073/pnas.0703766104

276. Oszvald M, Kang TJ, Tomoskozi S, Jenes B, Kim TG, Cha YS, et al. Expression of cholera toxin B subunit in transgenic rice endosperm. *Mol Biotechnol*. (2008) 40:261–8. doi: 10.1007/s12033-008-9083-2

277. Sharma MK, Singh NK, Jani D, Sisodia R, Thungapathra M, Gautam JK, et al. Expression of toxin co-regulated pilus subunit a (TCPA) of *Vibrio cholerae* and its immunogenic epitopes fused to cholera toxin B subunit in transgenic tomato (*Solanum lycopersicum*). *Plant Cell Rep*. (2008) 27:307–18. doi: 10.1007/s00299-007-0464-y

278. Tokuhara D, Yuki Y, Nochi T, Kodama T, Mejima M, Kurokawa S, et al. Secretory IgA-mediated protection against *V. cholerae* and heat-labile enterotoxin-producing enterotoxigenic *Escherichia coli* by rice-based vaccine. *Proc Natl Acad Sci U S A*. (2010) 107:8794–9. doi: 10.1073/pnas.0914121107

279. Nochi T, Yuki Y, Katakai Y, Shibata H, Tokuhara D, Mejima M, et al. A Rice-based Oral cholera vaccine induces macaque-specific systemic neutralizing antibodies but does Not influence pre-existing intestinal immunity. *J Immunol*. (2009) 183:6538–44. doi: 10.4049/jimmunol.0901480

280. Takeyama N, Yuki Y, Tokuhara D, Oroku K, Mejima M, Kurokawa S, et al. Oral rice-based vaccine induces passive and active immunity against enterotoxigenic *E. coli*-mediated diarrhea in pigs. *Vaccine*. (2015) 33:5204–11. doi: 10.1016/j.vaccine.2015.07.074

281. Tokuhara D. Challenges in developing mucosal vaccines and antibodies against infectious diarrhea in children. *Pediatr Int*. (2018) 60:214–23. doi: 10.1111/ped.13497

282. Yuki Y, Nojima M, Hosono O, Tanaka H, Kimura Y, Satoh T, et al. Oral MucoRice-CTB vaccine for safety and microbiota-dependent immunogenicity in humans: a phase 1 randomised trial. *Lancet Microbe*. (2021) 2:e429–40. doi: 10.1016/S2666-5247(20)30196-8

283. Liang W, Wang S, Yu F, Zhang L, Qi G, Liu Y, et al. Construction and evaluation of a safe, live, oral *Vibrio cholerae* vaccine candidate, IEM108. *Infect Immun*. (2003) 71:5498–504. doi: 10.1128/IAI.71.10.5498-5504.2003

284. Liu G, Yan M, Liang W, Qi G, Liu Y, Gao S, et al. Resistance of the cholera vaccine candidate IEM108 against CTXΦ infection. *Vaccine*. (2006) 24:1749–55. doi: 10.1016/j.vaccine.2005.09.059

285. Ledón T, Ferrán B, Pérez C, Suzarte E, Vichi J, Marrero K, et al. TLP01, an mshA mutant of *Vibrio cholerae* O139 as vaccine candidate against cholera. *Microbes Infect*. (2012) 14:968–78. doi: 10.1016/j.micinf.2012.04.004

286. Ravichandran M, Ali SA, Rashid NHA, Kurunathan S, Yean CY, Ting LC, et al. Construction and evaluation of a O139 *Vibrio cholerae* vaccine candidate based on a hemA gene mutation. *Vaccine*. (2006) 24:3750–61. doi: 10.1016/j.vaccine.2005.07.016

287. O'Ryan M, Vidal R, del Canto F, Salazar JC, Montero D. Vaccines for viral and bacterial pathogens causing acute gastroenteritis: Part I: overview, vaccines for enteric viruses and *Vibrio cholerae*. *Hum Vaccin Immunother*. (2015) 11:584–600. doi: 10.1080/21645515.2015.1011019

288. Pastor M, Pedraz JL, Esquisabel A. The state-of-the-art of approved and under-development cholera vaccines. *Vaccine*. (2013) 31:4069–78. doi: 10.1016/j.vaccine.2013.06.096

289. Altindis E, Fu Y, Mekalanos JJ. Proteomic analysis of *Vibrio cholerae* outer membrane vesicles. *Proc Natl Acad Sci U S A*. (2014) 111:E1548–56. doi: 10.1073/pnas.1403683111

290. Leitner DR, Lichtenegger S, Temel P, Zingl FG, Ratzberger D, Roier S, et al. A combined vaccine approach against *Vibrio cholerae* and ETEC based on outer membrane vesicles. *Front Microbiol*. (2015) 6:823. doi: 10.3389/fmicb.2015.00823

291. Schild S, Nelson EJ, Camilli A. Immunization with *Vibrio cholerae* outer membrane vesicles induces protective immunity in mice. *Infect Immun*. (2008) 76:4554–63. doi: 10.1128/IAI.00532-08

292. Bishop AL, Schild S, Patimalla B, Klein B, Camilli A. Mucosal immunization with *Vibrio cholerae* outer membrane vesicles provides maternal protection mediated by antilipopolysaccharide antibodies that inhibit bacterial motility. *Infect Immun*. (2010) 78:4402–20. doi: 10.1128/IAI.00398-10

293. Wang Z, Lazinski DW, Camilli A. Immunity provided by an outer membrane vesicle cholera vaccine is due to O-antigenspecific antibodies inhibiting bacterial motility. *Infect Immun*. (2017) 85:e00626-16. doi: 10.1128/IAI.00626-16

294. Buonaguro FM, Buonaguro L. Virus-like particles in vaccine development. *Virus Like Part Vaccine Dev*. (2014) 9:1–136. doi: 10.2217/9781780844176

295. Mohsen MO, Zha L, Cabral-Miranda G, Bachmann MF. Major findings and recent advances in virus–like particle (VLP)-based vaccines. *Semin Immunol*. (2017) 34:123–32. doi: 10.1016/j.smim.2017.08.014

296. Rashidijahanabad Z, Kelly M, Kamruzzaman M, Qadri F, Bhuiyan R, Mcfall-boegeman H, et al. Virus-like particle display of *Vibrio choleraeO*-specific polysaccharide as a potential vaccine against cholera. *ACS Infect Dis*. (2022) 8:574–83. doi: 10.1021/acsinfecdis.1c00585

297. Soria-Guerra RE, Moreno-Fierros L, Rosales-Mendoza S. Two decades of plant-based candidate vaccines: a review of the chimeric protein approaches. *Plant Cell Rep*. (2011) 30:1367–82. doi: 10.1007/s00299-011-1065-3

298. Montero DA, Del Canto F, Salazar JC, Céspedes S, Cádiz L, Arenas-Salinas M, et al. Immunization of mice with chimeric antigens displaying selected epitopes confers protection against intestinal colonization and renal damage caused by Shiga toxin-producing *Escherichia coli*. *NPJ Vaccines*. (2020) 5:20. doi: 10.1038/s41541-020-0168-7

299. Price GA, Holmes RK. Evaluation of TcpF-A2-CTB chimera and evidence of additive protective efficacy of immunizing with TcpF and CTB in the suckling mouse model of cholera. *PLoS One*. (2012) 7:e42434. doi: 10.1371/journal.pone.0042434

300. Price GA, Holmes RK. Immunizing adult female mice with a TcpA-A2-CTB chimera provides a high level of protection for their pups in the infant mouse model of cholera. *PLoS Negl Trop Dis*. (2014) 8:e3356. doi: 10.1371/journal.pntd.0003356

301. Zareitaher T, Sadat Ahmadi T, Latif Mousavi Gargari S. Immunogenic efficacy of DNA and protein-based vaccine from a chimeric gene consisting OmpW, TcpA and CtxB, of *Vibrio cholerae*. *Immunobiology*. (2022) 227:152190. doi: 10.1016/j.imbio.2022.152190

302. Upadhyay I, Li S, Ptacek G, Seo H, Sack DA, Zhang W. A polyvalent multiepitope protein cross-protects against *Vibrio cholerae* infection in rabbit colonization and passive protection models. *Proc Natl Acad Sci*. (2022) 119:2017. doi: 10.1073/pnas.2202938119

303. Sit B, Fakoya B, Waldor MK. Animal models for dissecting *Vibrio cholerae* intestinal pathogenesis and immunity. *Curr Opin Microbiol*. (2022) 65:1–7. doi: 10.1016/j.mib.2021.09.007

304. Raahati Z, Bakhshi B, Najar-Peerayeh S. Selenium nanoparticles induce potent protective immune responses against *Vibrio cholerae* WC vaccine in a mouse model. *J Immunol Res*. (2020) 2020:8874288. doi: 10.1155/2020/8874288

305. Albutti A, Longet S, McEntee CP, Quinn S, Liddicoat A, Rîmniceanu C, et al. Type ii nkt cell agonist, sulfatide, is an effective adjuvant for oral heat-killed cholera vaccines. *Vaccine*. (2021) 9:1–15. doi: 10.3390/vaccines9060619

306. Wang ZB, Xu J. Better adjuvants for better vaccines: progress in adjuvant delivery systems, modifications, and adjuvant–antigen codelivery. *Vaccine*. (2020) 8:128. doi: 10.3390/vaccines8010128

307. Subiza J, El-Qutob D, Fernandez-Caldas E. New developments in oral vaccines and mucosal adjuvants. *Recent Patents Inflamm Allergy Drug Discov*. (2015) 9:4–15. doi: 10.2174/1872213x09666150211122313

308. Jazayeri SD, Lim HX, Shameli K, Yeap SK, Poh CL. Nano and microparticles as potential oral vaccine carriers and adjuvants against infectious diseases. *Front Pharmacol*. (2021) 12:682286. doi: 10.3389/fphar.2021.682286

309. Chen W. Will the mRNA vaccine platform be the panacea for the development of vaccines against antimicrobial resistant (AMR) pathogens? *Expert Rev Vaccines*. (2022) 21:155–7. doi: 10.1080/14760584.2022.2011226

310. Mayer RL, Verbeke R, Asselman C, Aernout I, Gul A, Eggermont D, et al. Immunopeptidomics-based design of mRNA vaccine formulations against *Listeria monocytogenes*. *Nat Commun*. (2022) 13:6075. doi: 10.1038/s41467-022-33721-y

311. Kon E, Levy Y, Elia U, Cohen H, Hazan-Halevy I, Aftalion M, et al. An effective mRNA-LNP vaccine against the lethal plague bacterium. *bioRxiv*. (2022). doi: 10.1101/2022.08.07.503096

Frontiers in Medicine

Translating medical research and innovation into improved patient care

A multidisciplinary journal which advances our medical knowledge. It supports the translation of scientific advances into new therapies and diagnostic tools that will improve patient care.

Discover the latest Research Topics

See more →

Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact